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13. ABSTRACT ***(Maximum 200 Words)***

Ataxia Telangiectasia (A-T) is an autosomal recessive disease characterized by a progressive cerebellar ataxia, severe immune deficiencies, gonadal atrophy, telangiectases, increased risk for cancer, particularly lymphomas, and radiation sensitivity. Additionally, carriers are suspected to be prone to other cancers including breast cancer. We are studying the biochemical function of ATM the product of the gene mutated in A-T in Xenopus, a simple model system suitable for cell cycle and checkpoint studies. We have previously cloned Xenopus ATM (X-ATM), and characterized the protein.
We have described the pattern of expression of both X-ATM mRNA and protein throughout early development in Xenopus, a model system for vertebrate development. We have established a cell-free system derived from Xenopus extracts that recapitulates A DNA damage checkpoint in vitro. Under these conditions, we have demonstrated that double strand breaks (DSBs), the most harmful type of DNA damage can activate a checkpoint. The checkpoint is ATM-dependent and leads to the inactivation of cdk2 by phosphorylation on tyrosine 15. Inhibition of cdk2 prevents the loading of cdc45 on the origin of replication, thus inhibiting initiation of DNA replication. We also demonstrated that the checkpoint is independent of p53 activity.

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INTRODUCTION

Ataxia Telangiectasia (A-T) is an autosomal recessive disease which displays a complex phenotype [1, 2]. Patients exhibit a progressive cerebellar ataxia, in addition to severe immune deficiencies, gonadal atrophy, telangiectases, increased risk for cancer, particularly lymphomas, and radiation sensitivity. Additionally, carriers are suspected to be prone to other cancers including breast cancer [3-6].

Cells from A-T patients show increased radiosensitivity to ionizing radiation [7], increased chromosomal loss and breakage, and abnormal telomere morphology [8, 9]. Furthermore, these cells are defective in cell cycle checkpoints in G1, S and G2 phases of the cell cycle [10-12]. A striking cellular phenotype of A-T is the inability to prevent DNA replication following DNA damage also called Radioresistant DNA Synthesis (RDS). Although complex, the cellular phenotype of A-T points to a defect in handling DNA breaks formed either following damage or subsequent to normal physiological processes such as meiotic recombination and the maturation of the immune system.

Cells respond to DNA damage by activating checkpoint pathways that delay progression through the cell cycle [13, 14]. ATM functions in the checkpoint pathway activated by DNA damage.

Xenopus laevis is a powerful model system for both biochemical studies of cell cycle and checkpoint regulation as well as for developmental studies. We decided to use Xenopus as a novel model system to study both the biochemical role of ATM and its function during development. We have previously reported (1998 and 1999 reports) the cloning and characterization of the Xenopus homologue of ATM (X-ATM). This work was published last year [15].

The major objective of this proposal is to study the function of the ATM protein in a simple system in which biochemical analysis is a very powerful tool.

BODY

During the past year, we have finished the characterization of X-ATM by studying its spatiotemporal expression pattern during development. We have analyzed the patterns of expression of both X-ATM mRNA and protein. Our findings show that both mRNA and proteins switch from a uniform expression during early development to specific patterns as differentiation and organogenesis proceeds. In particular we found high levels of expression in both the developing somites and the developing eyes. A detailed description of our findings can be found in the appendices in an article that was published earlier this year [16].

We have developed a cell-free system that reconstitutes an ATM-dependent DNA damage checkpoint in vitro. This has been a major advance in our work and we anticipate that the benefits of this accomplishment will be very important. This is the first cell-free system in which to study DNA damage checkpoint. It will allow the detailed biochemical characterization of the signaling pathways that lead to cell cycle arrest following DNA damage. This will help understand how these pathways are altered in the case of cancer and breast cancer in particular and will provide critical information to design successful therapies. The detailed description of the cell-free system and of the checkpoint pathway we have discovered were recently published, the manuscript can be found in the appendices [17].

We have identified a DNA damage checkpoint pathway that prevents the initiation of DNA replication following DNA damage. This pathway is activated in vitro by double-strand breaks DSBs, the most harmful form of DNA damage. Signaling is dependent on ATM and is completely abolished by antibodies specific for X-ATM. Signaling leads to the inhibition of Cdk2/cyclinE activity. The inhibition of Cdk2 is due to the phosphorylation of

tyrosine residue 15. The inhibition of Cdk2 prevents the loading of cdc45 on the pre-replicative complex, preventing DNA replication initiation.

In addition, we have made significant progress towards the completion of objective #2 and #3. Because of the success we had in designing the cell-free extract described above, we have changed the strategy used to achieve these objectives, the objectives remaining the same. To study the interactions of X-ATM with other proteins, we are using a strategy that will uncover biologically relevant interactions. We originally proposed to use a two-hybrid screen in yeast to identify X-ATM partners. From the work performed in other laboratory in other species, this strategy turned out to be less productive than expected. We have shown that X-ATM can associate to DSBs in cell-free extracts (figure 1). We decided to use this observation to purify X-ATM-containing complexes and X-ATM-binding proteins. We have used this approach to show that X-ATM associates with the Xenopus homologue of MRE11 (X-MRE11), a gene important for DNA repair, DNA recombination and DNA damage response (figure 2).

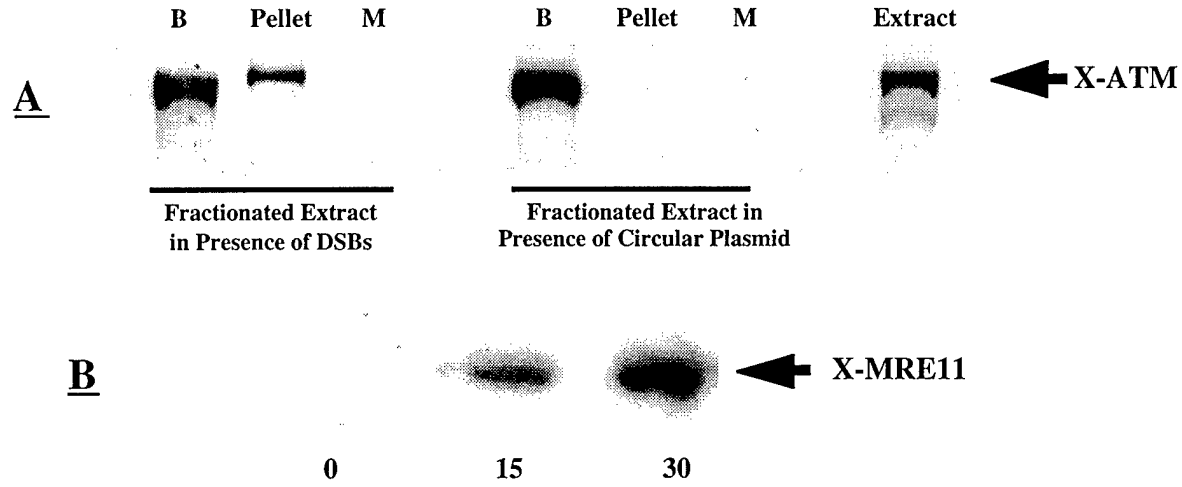

Figure 1: Association of X-ATM and X-MRE11 with DSBs in Xenopus extracts. A) X-ATM protein was traced by Western blot analysis throughout the extract fractionation procedure, either in the presence of DSBs-containing DNA (first 3 lanes) or in presence of circular plasmid (next 3 lanes). The right lane shows an interphase extract as control for electrophoretic mobility. When prepared in presence of DSBs, a fraction of X-ATM partitioned in the pellet along with the DSBs-containing DNA. B) A biotinylated double-strand oligonucleotide was coupled to neutravidin agarose beads *in vitro* then washed. The beads were incubated for 0, 15 or 30 minutes in interphase extracts derived from Xenopus eggs. The beads were washed extensively, then boiled in protein electrophoresis sample buffer and processed for electrophoresis followed by Western blotting using anti- X-MRE11 antibodies. The binding of MRE11 is evident after 15 minutes and increases with time. Control beads not coupled to oligonucleotides did not show any signal (data not shown).

We are currently studying the functional relationship between X-ATM and X-MRE11, and in particular we will determine if the DSBs-dependent phosphorylation of X-MRE11 we observe (figure 2) is dependent upon X-ATM.

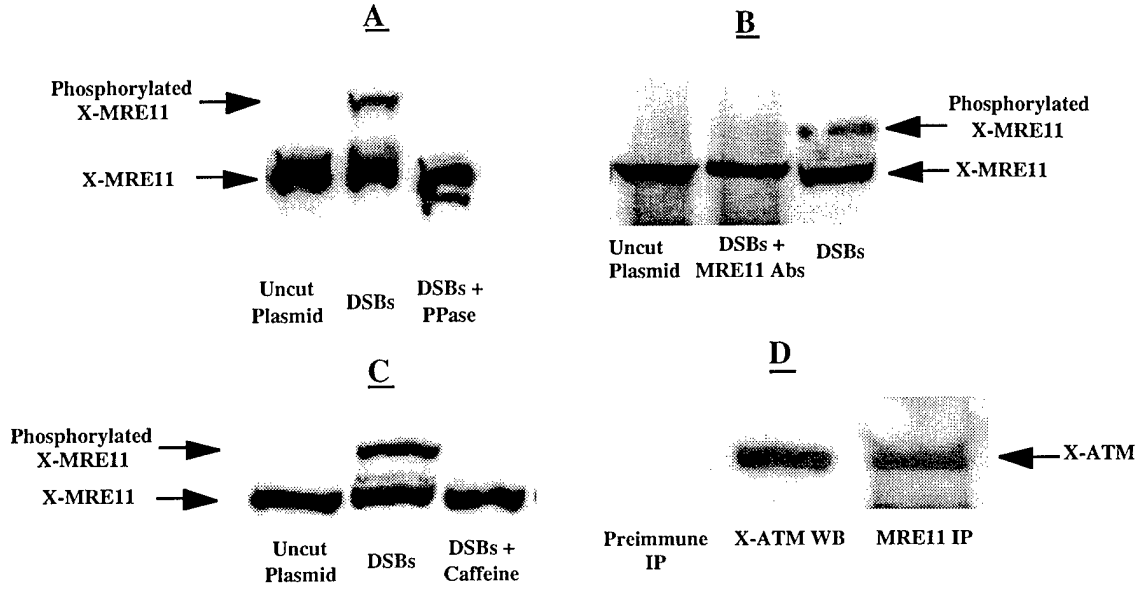

Figure 2: Xenopus MRE11. A) DSBs-dependent phosphorylation of MRE11 in Xenopus extracts. Western blot of X-MRE11 from extracts treated with uncut plasmid, DSBs-containing DNA or DSBs containing DNA followed by treatment with λ phosphatase, as indicated. B) Inhibition of X-MRE11 modification by anti-X-MRE11 antibodies. DSBs-induced phosphorylation of X-MRE11 is inhibited by incubation of the extract with anti-X-MRE11 antibodies (middle lane). C) DSBs-induced X-MRE11 phosphorylation is inhibited by Caffeine in the extract. Xenopus extract was incubated with DSBs (middle lane) or DSBs and 5mM caffeine (third lane). The mobility of X-MRE11 was determined by Western blot. D) Association of X-ATM and X-MRE11. Xenopus extracts were immunoprecipitate with anti-X-Mre11 antibodies (3rd lane) or with pre-immune antibodies (1st lane), followed by Western blotting with X-ATM antibodies. The middle lane shows the mobility of X-ATM in a direct Western blot.

Finally, we are staring to use the affinity of X-ATM for DSBs to purify novel proteins that are associating with X-ATM upon DNA damage.

KEY RESEARCH ACCOMPLISHMENTS

- Description of X-ATM spatio-temporal patterns of expression.
- Establishment of a Cell-free system that recapitulates a DNA damage checkpoint.
- Unravel a novel DNA damage checkpoint pathway that is ATM-dependent.
- Demonstrate interactions between ATM and MRE11, 2 genes mutated in cancer-prone syndromes

REPORTABLE OUTCOMES

Manuscripts:

Robertson, K., C. Hensey, and **J. Gautier**, Isolation and characterization of Xenopus ATM (X-ATM): expression, localization, and complex formation during oogenesis and early development. Oncogene, 1999. **18**(50): p. 7070-9.
Hensey, C., K. Robertson, and **J. Gautier**, Expression and Subcellular localization of X-ATM During Early Xenopus Development. Development, Genes and evolution, 2000. **210**: p. 467-469.
Costanzo, V., Robertson, R., Ying, C., Kim, K., Avvedimento, E., Gottesman, M., Grieco, D., and **Gautier, J.** Reconstitution of an ATM-dependent checkpoint that inhibits chromosomal DNA replication following DNA damage. Molecular Cell, 2000. **6**: p. 649-649.

Abstracts and presentations:

Novartis Conference: " Cell cycle and development". London, April 2000.
Era of Hope Meeting. Atlanta, June 2000.
8th International xenopus Conference. Estes Park (CO), August 2000.
DNA Replication Meeting, Salk Institute. San Diego, September,2000.

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CONCLUSIONS

We have been very successful in establishing a novel system to study DNA damage checkpoints in vitro. We believe that the implication of this work will be dramatic. It is the only in vitro system available to study DNA damage checkpoint signaling. It will allow to address questions that were not experimentally accessible in other model systems.
Although our approved objectives have not change, we propose to use a different strategy based on out finding to achieved the same goals. This change in strategy is described in the body of the proposal.
Finally, we have demonstrated that the use of a simple model system, Xenopus in this case, will help understand and reconcile data obtained in mammalian systems.

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APPENDICES:

The three manuscripts listed in the "Outcome" section are attached as appendices.

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Isolation and characterization of *Xenopus* ATM (X-ATM): expression, localization, and complex formation during oogenesis and early development

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ATM, the gene product mutated in Ataxia Telangiectasia (A-T) encodes a 350-kDa protein involved in the regulation of several cellular responses to DNA breaks. We used a degenerate PCR-based strategy to isolate a partial clone of X-ATM, the *Xenopus* homologue of human ATM. Sequence analysis and confirmed that the clone was most closely related to human ATM. *Xenopus* ATM protein (X-ATM) is 85% identical to human ATM within the kinase domain and 71% identical over the carboxyl-terminal half of the protein. Polyclonal antibodies raised against recombinant X-ATM are highly specific for the ATM protein and recognize a single polypeptide of 370-kDa in oocytes, embryos, egg extracts and a *Xenopus* cell line. We found that X-ATM was expressed maternally in eggs and as early as stage II pre-vitellogenic oocytes, and the protein and mRNA were present at relatively constant levels throughout development. Subcellular fractionation showed that the protein was nuclear in both the female and male germlines. The level of X-ATM protein did not change throughout the meiotic divisions or the synchronous mitotic cycles of cleavage stage embryos. In addition, we did not observe any change in the level or mobility of X-ATM protein following γ-irradiation of embryos. Finally, we also demonstrated that X-ATM was present in a high molecular weight complex of approximately 500 kDa containing the X-ATM protein and other, as yet unidentified component(s).

Keywords: xenopus; ATM; development; cell cycle; checkpoints

Introduction

Ataxia Telangiectasia (A-T) is an autosomal recessive disease which displays a complex phenotype (Boder and Sedgwick, 1970; Shiloh, 1998). Patients exhibit a progressive cerebellar ataxia, in addition to severe immune deficiencies, gonadal atrophy, telangiectases, increased risk for cancer, particularly lymphomas, and radiation sensitivity. Additionally, carriers are suspected to be prone to other cancers including breast cancer (Athma *et al.*, 1996; Chen *et al.*, 1998; Stankovic *et al.*, 1999; Swift *et al.*, 1999; Yuille and Coignet, 1998).

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Cells from A-T patients show increased radiosensitivity to ionizing radiation (Lavin and Shiloh, 1997), increased chromosomal loss and breakage, and abnormal telomere morphology (Smilenov *et al.*, 1997; Vaziri *et al.*, 1997). Furthermore, these cells are defective in cell cycle checkpoints in G1, S and G2 phases of the cell cycle (Beamish *et al.*, 1996; Hoekstra, 1997; Meyn, 1995). Although complex, the cellular phenotype of A-T points to a defect in handling DNA breaks formed either following damage or subsequent to normal physiological processes such as meiotic recombination and the maturation of the immune system.

The identification of a single mutated gene called ATM (Ataxia Telangiectasia Mutated) as the molecular basis for the phenotype has allowed a better understanding of both ATM function and the A-T pleiotropic phenotypes (Savitsky *et al.*, 1995; Taylor, 1998). ATM is a nuclear phosphoprotein (Chen and Lee, 1996; Scott *et al.*, 1998) and is a Serine/Threonine protein kinase for which the c-abl proto-oncogene, the replication protein RPA and the p53 tumor-suppressor gene have been identified as substrates *in vitro* (Banin *et al.*, 1998; Baskaran *et al.*, 1997; Canman *et al.*, 1998; Gately *et al.*, 1998; Khanna *et al.*, 1998; Nakagawa *et al.*, 1999; Shafman *et al.*, 1997). The identification of these potential substrates have helped place ATM in a signal transduction pathway in which it could function in a cell cycle checkpoint through c-abl and p53.

Cells respond to DNA damage by activating checkpoint pathways that delay progression through the cell cycle (Hensey and Gautier, 1995). ATM functions in the checkpoint pathway activated by DNA damage. It has been shown that p53 is phosphorylated in an ATM-dependent fashion following DNA damage suggesting that p53 could be a direct substrate for the ATM protein kinase (Banin *et al.*, 1998; Canman *et al.*, 1998). Activation of p53 following DNA damage also involves dephosphorylation events that are dependent upon ATM activity (Waterman *et al.*, 1998). In addition, studies in yeast and in mammalian cells also suggest that ATM might function as an upstream regulator of the Chk1 and Chk2 kinases (Brown *et al.*, 1999; Chen *et al.*, 1999; Matsuoka *et al.*, 1998). However, the exact *in vivo* biochemical function of the ATM protein and its physiological substrates still remain elusive. In particular, the gap between our *in vitro* biochemical knowledge [illegible] patient phenotypes still awaits investigation.

A mouse model for ATM deficiency was created in several laboratories by specific germline inactivation of

the ATM gene (Barlow *et al*., 1996; Elson *et al*., 1996; Herzog *et al*., 1998; Xu *et al*., 1996; Xu and Baltimore, 1996). Fibroblasts isolated from ATM$^{-/-}$ mice display similar cellular phenotypes to those observed in cells from A-T patients. As is the case with A-T patients, ATM deficient mice display a variety of growth defects, meiotic defects, immunological abnormalities, acute radiation sensitivity and cancer predisposition, confirming the pleiotropic roles of ATM.

Xenopus laevis is a powerful model system for both biochemical studies of cell cycle and checkpoint regulation as well as for developmental studies. We decided to use *Xenopus* as a novel model system to study both the biochemical role of ATM and its function during development. The influence of DNA replication inhibition upon cell cycle progression (DNA replication or S phase checkpoint) has been studied in detail in *Xenopus* egg extracts. First, it has been shown that threshold inhibition of DNA replication can elicit cell cycle arrest in egg extracts and prevent entry into mitosis (Dasso and Newport, 1990). Furthermore, both cdc25 and wee1 have been identified as modulators of this checkpoint. Upstream of cdc25, the protein kinase Chk1 and the 14-3-3 adapter protein have also been shown to be essential for cell cycle arrest (Kumagai *et al*., 1998a,b).

To enable such studies we have obtained a partial clone of the *Xenopus* homolog of the ATM protein. We present its detailed expression patterns during the meiotic and the mitotic cell cycles as well as during development. We show that X-ATM is a maternal protein expressed as early as stage II oocytes and that ATM is exclusively nuclear in the germline. Finally, we present evidence that ATM exists in a large molecular weight complex in extracts derived from eggs.

Results

Isolation of a partial clone of the Xenopus *homolog of ATM*

A protein alignment of the putative kinase domain of human ATM, *S. pombe* rad3, human ATR, *S. cerevisiae* TEL1, and *D. melanogaster* mei41 was used to identify regions of homology within the kinase domain of ATM and TEL1, that were not present in ATR, rad3 or mei41. Four amino acid stretches were identified and used to design degenerate oligonucleotides (see Materials and methods, Figure 1b). A 480 bp cDNA fragment was isolated by RT–PCR (Figure 1a) and sequencing of the fragment confirmed that it was most closely related to human ATM (Figure 1b). Using library screening, ATM-specific library construction, and 5′RACE (Figure 1a) additional sequence corresponding to approximately 50% of the predicted *Xenopus* ATM ORF was obtained.

An alignment of the carboxyl-terminal half of X-ATM with human ATM is shown in Figure 1b. ATM is highly conserved between *Xenopus* and human with an overall identity of 71% in the region we have sequenced. Within the kinase domain the identity is 85% while the region upstream of the kinase domain (amino acids 1–1100 of X-ATM) shows 66% identity to the human protein. In contrast, when this region from X-ATM (a.a. 1–1100) is aligned with *S.c.* TEL1, *S.p.* rad3, *D.m.* mei41 or *H.s.* ATR, only short stretches of weak homology (ranging from 20–24%) are detected.

Characterization of X-ATM antibodies

Using an *E. coli* expression vector, we produced a 20-kDa fragment of the X-ATM cDNA corresponding to the region immediately 5′ of the protein kinase domain (PQE60/TBH6H of Figure 1a). We chose this region as it had been previously shown to elicit a good immune response for human ATM (Brown *et al*., 1997). Additionally, we wanted to avoid raising antibodies against the conserved kinase domain which could potentially cross-react with other members of the ATM/PI3 kinase family. This 20-kDa polypeptide was expressed in *E. coli* and was used as an antigen for antibody production in rabbits (see Materials and methods).

Programmed reticulocyte lysate containing the 65-kDa carboxyl terminal portion of X-ATM (T7/TBH-XTC of Figure 1a) was used to screen the different antisera. Sera from both rabbits showed strong cross-reactivity with the translated product (data not shown) and recognized a protein of approximately 370-kDa in *Xenopus* egg extracts (Figure 2a, lanes 1, 2). Due to the presence of a 210-kDa cross-reacting polypeptide in serum A (Figure 2a, lane 1), we used serum B for the remaining experiments. Preincubation of the antiserum with the 20-kDa polypeptide used for immunization eliminated the signal, confirming the identity of the protein recognized by the antiserum (data not shown). Moreover, we were able to immunoaffinity purify the X-ATM antibody from rabbit B using the 20-kDa polypeptide coupled to agarose. This purified antibody, which was used in all further experiments, detected a single polypeptide in *Xenopus* egg extracts that migrated slightly slower than human ATM (compare lanes 3 and 4, Figure 2a). It also immunoprecipitated a fraction of the X-ATM protein from extracts as shown in Figure 2b (lane 2).

Expression of X-ATM during early development

Little is known about ATM expression during the early phases of vertebrate development. We therefore examined the temporal expression of both X-ATM mRNA and protein between fertilization and the swimming tadpole stage in *Xenopus*. Figure 3a shows an RNAse protection assay for the XTC cell line (Pudney *et al*., 1973) and the indicated stages of development. X-ATM mRNA was detected in unfertilized eggs and expressed at relatively constant levels throughout cleavage, gastrulation, neurulation and the tadpole stage, although we consistently detected higher X-ATM mRNA levels in unfertilized eggs. Since transcription of zygotic genes does not start until the midblastula transition (St. 8-9), this demonstrates that X-ATM mRNA is maternally inherited. The temporal expression of X-ATM protein during similar developmental stages is shown in Figure 3b. The protein is expressed maternally and throughout development where we observe a single cross-reacting polypeptide at all stages. Some varia-

bility in the X-ATM protein amounts was detected, particularly during neurulation. This might reflect developmentally regulated changes in the amount of X-ATM. Alternatively, this could also be due to the subcellular localization of the protein leading to different yields following embryonic extract preparation. We estimated the concentration of X-ATM to be 200 nM in egg extracts and 40 nM in unfertilized eggs (see Materials and methods).

Subcellular localization of the ATM protein in the germline

X-ATM is present in egg extracts and in unfertilized eggs (Figures 2a and 3b). We examined whether the protein was already present in the oocyte as opposed to being synthesized during meiotic maturation as has been described for some proteins (Sagata *et al.*, 1988). *Xenopus* oocytes were manually dissected from pieces of ovary and protein extracts were made at the stages of oogenesis indicated (Dumont, 1972). X-ATM is already present in previtellogenic oocytes (stage II) and persists throughout oogenesis (Figure 4a). Although the concentration of X-ATM per mass of tissue does not vary significantly throughout oogenesis, the mass per oocyte increases dramatically since the volume of the oocytes increases by a factor of 10^3 between stages I–II and stage VI.

Stage VI *Xenopus* oocytes contain a large (200 μm diameter) nucleus, known as the germinal vesicle (GV), which can be used to store nuclear proteins important for later development (Dreyer and Hausen, 1983). The GV can be isolated surgically from its surrounding cytoplasm, allowing the preparation of pure cytoplasmic and nuclear fractions from oocytes (Ford and Gurdon, 1977). We demonstrate that X-ATM is entirely nuclear in stage VI oocytes (Figure 4b). Moreover, using either demembranated sperm nuclei, or a nuclear extract prepared from these nuclei following their incubation in extracts (Walter *et al.*, 1998), we show that X-ATM is also nuclear in the male germline.

Expression of X-ATM protein throughout the meiotic and mitotic cell cycles

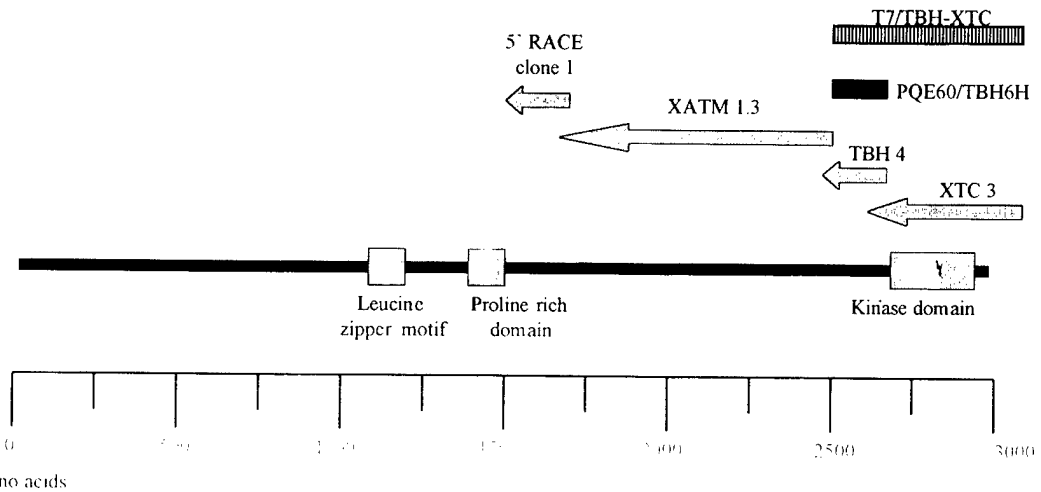
In *Xenopus* oocytes the highly synchronous meiotic cell cycle is easily studied *in vitro*, where progesterone induced oocyte maturation culminates in the completion of meiosis I and the progression to metaphase of meiosis II. To determine whether X-ATM protein levels fluctuated during the meiotic cell cycle, we performed Western blots on protein samples from oocytes synchronously undergoing meiosis. X-ATM protein levels remained constant throughout meiosis (Figure 5a). In this experiment, prophase-arrested oocytes were induced to undergo meiotic maturation *in vitro* by progesterone. Nuclear envelope breakdown took place at 3.5 h, meiosis I occurred at 6 h and the oocytes were arrested in metaphase of meiosis II by 9 h.

Next, we followed X-ATM levels throughout the mitotic cell cycle which oscillations were assessed by following cdc2/cyclinB activity. The naturally synchronous cell divisions of the early embryo allow the preparation of cell free extracts that undergo cell cycle oscillations *in vitro*. Using such extracts we examined X-ATM protein levels throughout these highly synchronized cell cycles. ATM is expressed throughout the cell cycle and its levels remain constant over the course of two cell cycles (Figure 5b). Additionally, there was no change in the electrophoretic mobility of X-ATM.

X-ATM protein in γ-irradiated eggs

In *Xenopus*, irradiation of cleavage stage embryos leads to massive and synchronous apoptosis at the onset of gastrulation (St. 10.5) (Anderson *et al.*, 1997; Hensey and Gautier, 1997). To assess whether this response correlated with changes in the amounts or the electrophoretic mobility of X-ATM, we compare the levels of X-ATM in protein samples from untreated and γ-irradiated embryos between stage 8 and 10.5. X-ATM protein levels were similar in control and irradiated embryos. In addition no change in the mobility of the protein was detected (Figure 6).

A



B

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X-ATM      DVSTRSLLLC LDLLHRVCRA AVTHCEDALE CHHHVIVGSL   40    X-ATM      MFQIKQYNPS QLGVSEWQLE EAQIFWDKEE PSLAREILKQ   840
H-ATM      DVSLRSFSLC CDLLSQVCQT AVTYCKDALE NHLHVIVGTL 1520    H-ATM      IFQIKQYNSV SCGVSEWQLE EAQVFWAKKE QSLALSILKQ  2310
Consensus  DVS.RS..LC .DLL..VC.. AVT.C.DALE .H.HVIVG.L         Consensus  .FQIKQYN.. ..GVSEWQLE EAQ.FW.K.E .SLA..ILKQ

X-ATM      IPLANSQESI QEKVCDLLNF LVIENKDNEN LYHTIKLLDP   80    X-ATM      MINKLEIKSF EVENDSRLRL LYAESLRLCG KWLAETCLES   880
H-ATM      IPLVYEQVEV QKQVLDLLKY LVIDNKDNEN LYITIKLLDP 1560    H-ATM      MIKKLDASC- -AANNPSLKL TYTECLRVCG NWLAETCLEN  2348
Consensus  IPL...Q... Q..V.DLL.. LVI.NKDNEN LY.TIKLLDP         Consensus  MI.KL..... ...N...L.L .Y.E.LR.CG .WLAETCLE.

X-ATM      FFDIPLFKNL RQAHQKIKYS KGPFSLLKEI QNFLSVSVCD   120   X-ATM      PTVIMQNYLE KAVEFAG-YS DGAGEKLQEG RMKAFLSLAR   919
H-ATM      FFDHVVFKDL RITQQKIKYS RGPFSLLEEI NHFLSVSVYD 1600    H-ATM      PAVIMQTYLE KAVEVAGNYD GESSDELRNG KMKAFLSLAR  2388
Consensus  FFD...FK.L R...QKIKYS .GPFSLL.EI ..FLSVSV.D         Consensus  P.VIMQ.YLE KAVE.AG.Y. ......L..G .MKAFLSLAR

X-ATM      SLPLTRLEGL NDLRKQLEQH KDQIKELVRD CQGTPQDSVI   160   X-ATM      FSDAQYQRID NYMKSSEFEN KQALLRKAKE EVGLIKQHKV   959
H-ATM      ALPLTRLEGL KDLRRQLELH KDQMVDIMRA SQDNPQDGIM 1640    H-ATM      FSDTQYQRIE NYMKSSEFEN KQALLKRAKE EVGLLREHKI  2428
Consensus  .LPLTRLEGL .DLR.QLE.H KDQ.....R. .Q..PQD...         Consensus  FSD.QYQRI. NYMKSSEFEN KQALL..AKE EVGL...EK.

X-ATM      ASLVVNLLQL SKNAVHQSNG KEVLEAVGSC LGELGPIDFS   200   X-ATM      QNNRYTVKVE RELQLDECAI LALREDRKRF LCKAVENYIS   999
H-ATM      VKLVVNLLQL SKMAINHTGE KEVLEAVGSC LGEVGPIDFS 1680    H-ATM      QTNRYTVKVQ RELELDELAL RALKEDRKRF LCKAVENYIN  2468
Consensus  ..LVVNLLQL SK.A..... KEVLEAVGSC LGE.GPIDFS         Consensus  Q.NRYTVKV. RELLDE.A. .AL.EDRKRF LCKAVENYI.

X-ATM      NIALQQHKKD SVYLKADKVF EEKELQCVLV MLTLINNALT   240   X-ATM      CLVSGEEHDM WIFRLCSLWL ENSAVSDVNS MMRQDAQKIP  1039
H-ATM      TIAIQHSKDA S-YTKALKLF EDKELQWTFI MLTYLNNTLV 1719    H-ATM      CLLSGEEHDM WVFRLCSLWL ENSGVSEVNG MMKRDGMKIP  2508
Consensus  .IA.Q..K.. S.Y.KA.K.F E.KELQ.... MLT..NN.L.         Consensus  CL.SGEEHDM W.FRLCSLWL ENS.VS.VN. MM..D..KIP

X-ATM      DHCIQVRSAA ATCLKNILAT KTGAMFWEAC KDKGEPMLLY   280   X-ATM      SHKFLPLMYQ LAARMGTKKM GNPGFHDVLN NLIGRISMDH  1079
H-ATM      EDCVKVRSAA VTCLKNILAT KTGHSFWEIY KMTTDPMLAY 1759    H-ATM      TYKFLPLMYQ LAARMGTKMM GGLGFHEVLN NLISRISMDH  2548
Consensus  ..C..VRSAA .TCLKNILAT KTG..FWE.. K....PML.Y         Consensus  ..KFLPLMYQ LAARMGTK.M G..GFH.VLN NLI.RISMDH

X-ATM      LQPFRAPKKK FLEVEEIQRE GALESLDDPG IWIPQNENHE   320   X-ATM      PHHTLFIILA LANANKDDQL MKAEAVKRSF LTKNAPKQIS  1119
H-ATM      LQPFRTSRKK FLEVPRFDKE NPFEGLDDIN LWIPLSENHD 1799    H-ATM      PHHTLFIILA LANANRDEFL TKPEVARRSR ITKNVPKQSS  2588
Consensus  LQPFR...KK FLEV.....E ...E.LDD.. .WIP..ENH.         Consensus  PHHTLFIILA LANAN.D..L .K.E...RSR .TKN.PKQ.S

X-ATM      SWIKHLTCTL LESGGVKSEV LLLLKPMCEV KADFCQAVVP   360   X-ATM      QLDKERMEAA RHIVDTIKKR RTDMVRDVER LCDAYITLAN  1159
H-ATM      IWIKTLTCAF LDSGGTKCEI LQLLKPMCEV KTDFCQTVLP 1839    H-ATM      QLDEDRTEAA NRIICTIRSR RPQMVRSVEA LCDAYIILAN  2628
Consensus  .WIK.LTC.. L.SGG.K.E. L.LLKPMCEV K.DFCQ.V.P         Consensus  QLD..R.EAA ..I..TI..R R..MVR.VE. LCDAYI.LAN

X-ATM      YIVHNILLND SNQTWRTLLS KNVQRFFTSC CRSLPSSSRS   400   X-ATM      MDANQWKSQR NAIPIPSDQP ITKLNKLHDV VIPTMEIKVD  1199
H-ATM      YLIHDILLQD TNESWRNLLS THVQGFFTSC LRHFSQTSRS 1879    H-ATM      LDATQWKTQR KGINIPADQP ITKLKNLEDV VVPTMEIKVD  2668
Consensus  Y..H.ILL.D .N..WR.LLS ..VQ.FFTSC .R.....SRS         Consensus  .DA.QWK.QR ..I.IP.DQP ITKL..L.DV V.PTMEIKVD

X-ATM      ATPASSDSES EGVARGAVDI ASRRTMLTMV EHLRKQRRPV   440   X-ATM      PSGEYENLVT IVSFKPEFRL AGGLNLPKII DCVGSDGKER  1239
H-ATM      TTPANLDSES EHFFRCCLDK KSQRTMLAVV DYMRRQKRPS 1919    H-ATM      HTGEYGNLVT IQSFKAEFRL AGGVNLPKII DCVGSDGKER  2708
Consensus  .TPA..DSES E...R...D. .S.RTML..V ...R.Q.RP.         Consensus  ..GEY.NLVT I.SFK.EFRL AGG.NLPKII DCVGSDGKER

X-ATM      SGTAFDDNFW LDLNYLEVAM AVQSCAAHFT ALLYSEIYTD   480   X-ATM      RQLVKGQDDL RQDAVMQQVF QMCNTLLQRN SETRKRKLTI  1279
H-ATM      SGTIFNDAFW LDLNYLEVAK VAQSCAAHFT ALLYAEIYAD 1959    H-ATM      RQLVKGRDDL RQDAVMQQVF QMCNTLLQRN TETRKRKLTI  2748
Consensus  SGT.F.D.FW LDLNYLEVA. ..QSCAAHFT ALLY.EIY.D         Consensus  RQLVKG.DDL RQDAVMQQVF QMCNTLLQRN .ETRKRKLTI

X-ATM      KVKQDGEQRT SANRSNARKC LKFEEGSQTL DITGLSEKSK   520   X-ATM      RRYKVVPLSH RSGVLEWCTG TVPIGEYLVN DKDGAHKRYR  1319
H-ATM      KKSMDDQE-- -------KRS LAFEEGSQST TISSLSEKSK 1990    H-ATM      CTYKVVPLSQ RSGVLEWCTG TVPIGEFLVN NEDGAHKRYR  2788
Consensus  K...D..... .......... L.FEEGSQ.. .I..LSEKSK         Consensus  ..YKVVPLS. RSGVLEWCTG TVPIGE.LVN ..DGAHKRYR

X-ATM      EETGISLQDL LMDIYRSIGE PDSLYGCGGG KMLHPLARIR   560   X-ATM      PGDYGSLQCQ RKMMEVQRGR FEEKYQMFLN VCENFRPVFR  1359
H-ATM      EETGISLQDL LLEIYRSIGE PDSLYGCGGG KMLQPITRLR 2030    H-ATM      PNDFSAFQCQ KKMMEVQKKS FEEKYEVFMD VCQNFQPVFR  2828
Consensus  EETGISLQDL L..IYRSIGE PDSLYGCGGG KML.P..R.R         Consensus  P.D....QCQ .KMMEVQ... FEEKY..F.. VC.NF.PVFR

X-ATM      TYEHEAKWGK ALVTFDLEMN LPPVTRQAGI MQALQNFGLC   600   X-ATM      YFCMEKFLDP AMWFEKRLAY TRSVATSSIV GYIVGLGDRH  1399
H-ATM      TYEHEAMWGK ALVTYDLETA IPSSTRQAGI IQALQNLGLC 2070    H-ATM      YFCMEKFLDP AIWFEKRLAY TRSVATSSIV GYILGLGDRH  2868
Consensus  TYEHEA.WGK ALVT.DLE.. .P..TRQAGI .QALQN.GLC         Consensus  YFCMEKFLDP A.WFEKRLAY TRSVATSSIV GYI.GLGDRH

X-ATM      HMLSTYLRGL EHENAEWSSE LQEIHFQAAW RNMQWDDSLP   640   X-ATM      VQNILIDEES AELVHIDLGV AFEQGKILPT PETVPFRLTR  1439
H-ATM      HILSVYLKGL DYENKDWCPE LEELHYQAAW RNMQWDHCTS 2110    H-ATM      VQNILINEQS AELVHIDLGV AFEQGKILPT PETVPFRLTR  2908
Consensus  H.LS.YL.GL ..EN..W..E L.E.H.QAAW RNMQWD....         Consensus  VQNILI.E.S AELVHIDLGV AFEQGKILPT PETVPFRLTR

X-ATM      TKNETSGPGY HESLYRAVQS LRDKEFCGFH DHIKYARVKE   680   X-ATM      DIVDGMGITG VEGVFRRCCE KTMEVMRNNQ EALLTIVEVL  1479
H-ATM      VSKEVEGTSY HESLYNALQS LRDREFSTFY ESLKYARVKE 2150    H-ATM      DIVDGMGITG VEGVFRRCCE KTMEVMRNSQ EFLLTIVEVL  2948
Consensus  ...E..G..Y HESLY.A.QS LRD.EF..F. ...KYARVKE         Consensus  DIVDGMGITG VEGVFRRCCE KTMEVMRN.Q E.LLTIVEVL

X-ATM      VEELCSGSLE SVYSLYPALC RLQAIGELAS VGQMFSRSIT   720   X-ATM      LYDPLFDWTM NPLKALYLQQ ---DEVDLNA TLGGDDPECN  1516
H-ATM      VEEMCKRSLE SVYSLYPTLS RLQAIGELES IGELFSRSVT 2190    H-ATM      LYDPLFDWTM NPLKALYLQQ RPEDETELHP TLNADDQECK  2988
Consensus  VEE.C..SLE SVYSLYP.L. RLQAIGEL.S .G..FSRS.T         Consensus  LYDPLFDWTM NPLKALYLQQ ...DE..L.. TL..DD.EC.

X-ATM      DDGLKDTFLK WQRQSQLLKD SDFEFLEPVL SLRSVILETL   760   X-ATM      RNSCD-SQSV NKVAERVLLR LQEKLTGVEE GMVLSVGGQV  1555
H-ATM      HRQLSEVYIK WQKHSQLLKD SDFSFQEPIM ALRTVILEIL 2230    H-ATM      RNLSDIDQSF DKVAERVLMR LQEKLKGVEE GTVLSVGGQV  3028
Consensus  ...L.....K WQ..SQLLKD SDF.F.EP.. .LR.VILE.L         Consensus  RN..D..QS. .KVAERVL.R LQEKL.GVEE G.VLSVGGQV

X-ATM      LQEKKQPSQ ESLKDILTKH LLDLSRIARS AGNTQLPEKA   800   X-ATM      NHLIQQAMDP KNLSRLFPGW KAWVZ      1580
H-ATM      MEKEMDNSQR ECIKDILTKH LVELSILART FKNTQLPERA 2270    H-ATM      NLLIQQAIDP KNLSRLFPGW KAWV-       305
Consensus  ...E..... E..KDILTKH L..LS..AR. ..NTQLPE.A         Consensus  N.LIQQA.DP KNLSRLFPGW KAWV.
```

Figure 1 (**a**) ATM domains and cloning strategy. The human ATM protein is represented by a thick black line, specific domains are indicated. X-ATM clones are indicated by arrows. T7/TBH-XTC denotes the *in vitro* translated fragment of X-ATM used to screen sera from rabbits A and B. PQE60 TBH6H was used in antigen production and part of this region was used in RNAse protection assays (construct T7/TBH-XTC). (**b**) Sequence comparison of X-ATM and human ATM. Fifty per cent of the predicted sequence of *Xenopus* ATM (X-ATM) was aligned with the amino acid sequence of human ATM (H-ATM) using Geneworks software (Intellegenetics). Identical amino acids between the two proteins are indicated in bold letters below the comparison. The regions of the human sequence that were used to derive degenerate oligonucleotides are underlined (thin lines). The PI3 kinase domain consensus region is underlined (thick line). The nucleotide sequence that was used to translate X-ATM has been deposited to GenBank, accession number AF174488

X-ATM is present in a protein complex in Xenopus *extracts*

The carboxyl-terminal kinase domain of ATM represents a small portion of this large protein. Little is known about the function of the domain(s) outside the kinase region but support for its importance comes from both genetic analysis of ATM mutants (Concannon and Gatti, 1997) as well as the finding that a putative leucine zipper region of ATM behaved as a transdominant negative (Morgan *et al*., 1997). A central proline rich domain has already been shown to interact with the c-abl SH3 domain (Shafman *et al*., 1997), and the remainder of the protein is suspected of playing regulatory functions by interacting with other

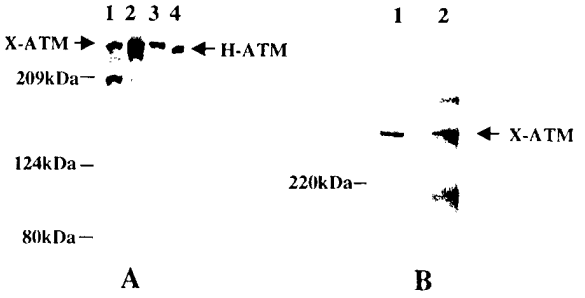

Figure 2 Characterization and specificity of antibodies against X-ATM. (**a**) Specificity of the crude serum and the affinity purified antibody for X-ATM. One hundred μg of *Xenopus* egg extract was electrophoresed (6% SDS–PAGE) and Western blotted with the crude serum A (lane 1), the crude serum B (lane 2) or the affinity purified antibody from serum B (lane 3). In all cases a polypeptide of 370 kDa was detected. Lane 4 is a human cell extract (HeLa) probed with an anti-human ATM antibody (Gately *et al*., 1998). (**b**) Immunoprecipitation of native X-ATM protein. Five hundred μg of extract were subjected to immunoprecipitation followed by Western blotting using the X-ATM antibody (lane 2). A 370 kDa polypeptide of the same size as that in the control extract (lane 1) is detected

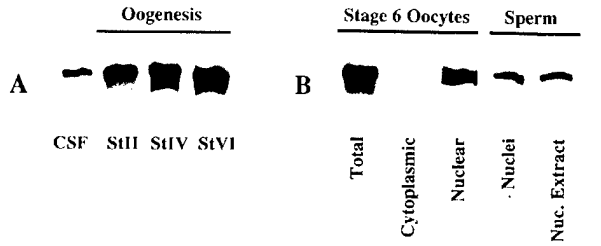

Figure 4 (**a**) X-ATM during oogenesis. Manually dissected oocytes at different stages of vitellogenesis were collected. Equal amounts of protein were separated by electrophoresis and subjected to Western blotting. The stages of oogenesis are indicated below the lanes. The mobility was compared with that of X-ATM in a CSF extract (CSF). (**b**) Subcellular localization of ATM in the germline. Protein samples from either complete stage VI oocytes (total), stage VI oocyte cytoplasm (cytoplasm) or stage VI oocyte nuclei (nuclear) were processed for Western blotting. The two right lanes show a similar Western blot using either demembranated sperm nuclei or protein extracts prepared from nuclei

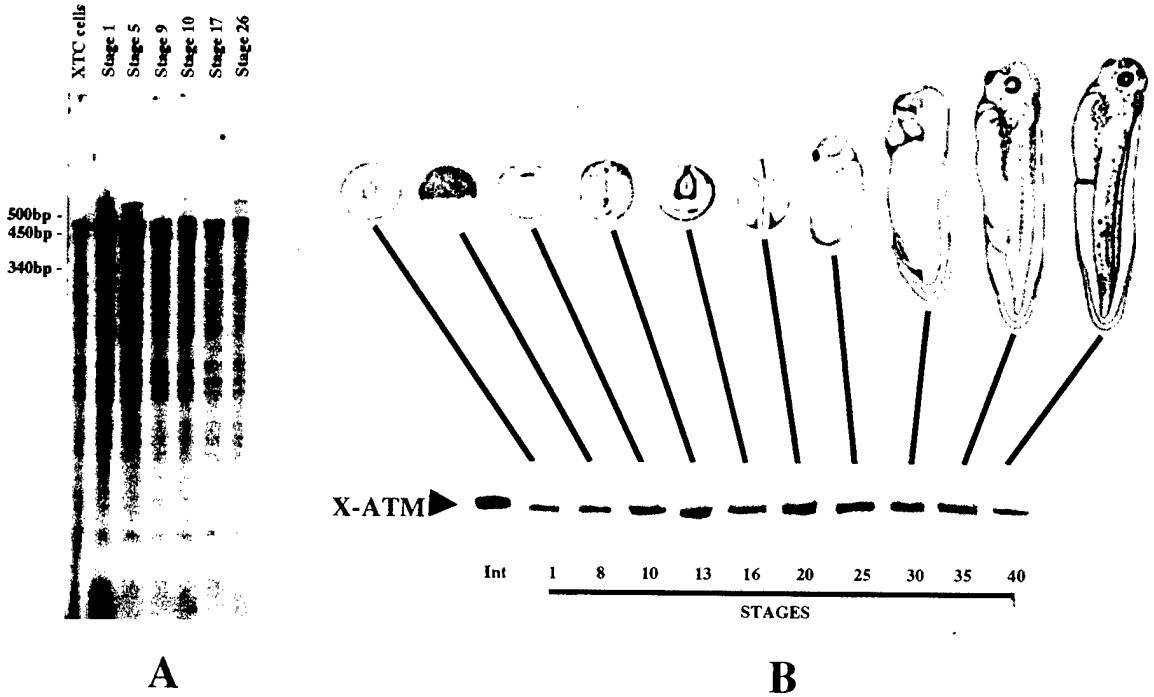

Figure 3 (**a**) Expression of X-ATM mRNA during development. RNAse protection assays using a 480 bp probe from clone TBH4 of the X-ATM cDNA were performed using standard protocols at different stages of development (as indicated above the lanes) and from RNA prepared from the *Xenopus* XTC cell line (1st lane). The protected band is 480 bp. (**b**) Expression of X-ATM protein during development. Equal amounts of protein extracts prepared from different stages were separated by electrophoresis followed by Western blot with the ATM affinity purified antibody. The protein is detected from stage 1 and the level of expression increases slightly through gastrulation (St. 13), and peaks at the time of formation of the neural tube and somites (St. 15–25), to slowly decrease thereafter. Drawings of the embryonic stages are shown above the autoradiogram

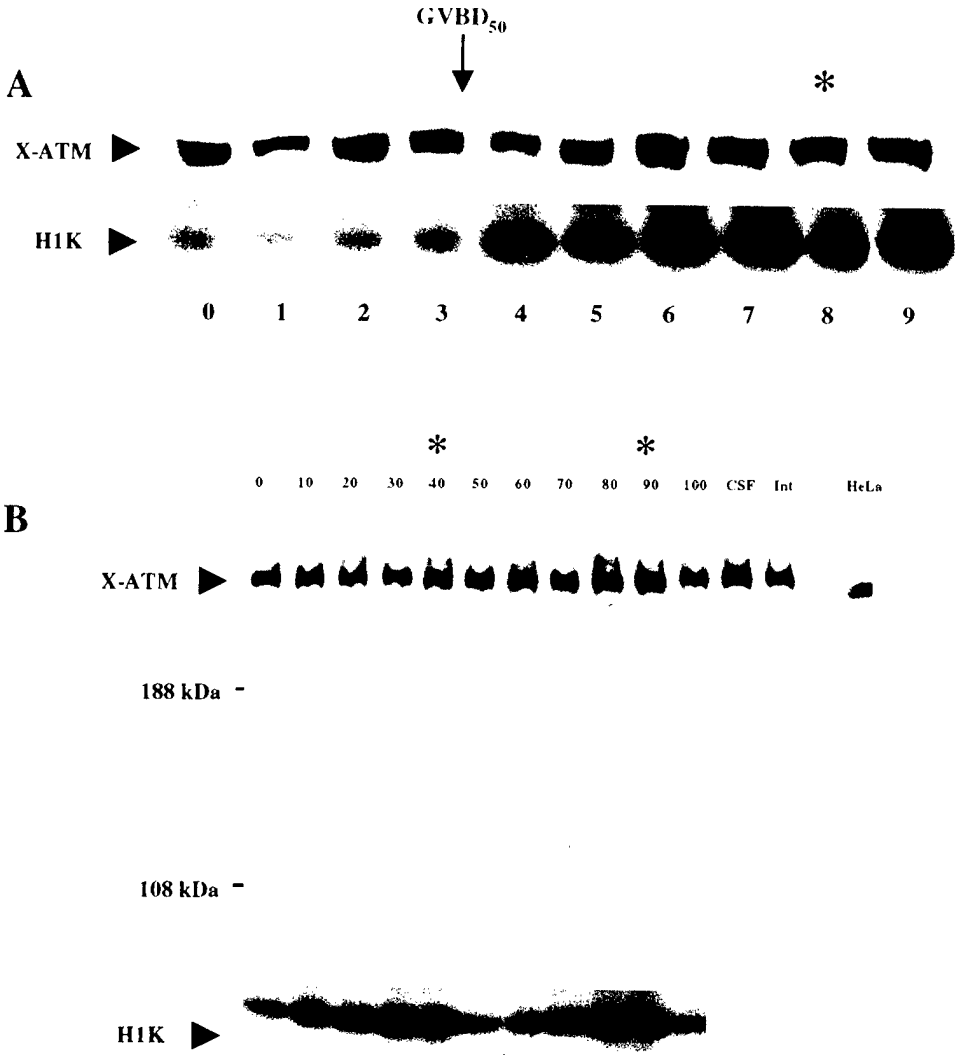

Figure 5 (a) X-ATM expression throughout the meiotic cell cycle. Western blot analysis of oocytes progressing synchronously through meiosis was performed. $t=0$ is the time of addition of progesterone. $GVBD_{50}$ indicates the G2/M transition, the *indicates the interphase between metaphase of meiosis I and metaphase of meiosis II. X-ATM Western blot: top panel, Histone H1 kinase activity of the same samples: bottom panel. (b) X-ATM during the mitotic cell cycles. ATM levels during S phase and mitosis were compared by Western blot analysis of 'cycling extracts' prepared from *Xenopus* eggs. X-ATM levels were monitored by 6% SDS–PAGE and Western blot analysis of extracts over a period of 100 min (top panel), corresponding to two cell cycles as determined by analysis of cdc2/cyclin B kinase activity (bottom panel). M phase, i.e. the kinase activity peaks, is indicated by *. Comparison of a mitotic extract (CSF), and an interphase extract (INT), also indicated a similar amount of ATM in these different cell cycle phases. For comparison, a HeLa whole-cell lysate was also analysed on this gel. Human ATM antibody (Gately *et al*., 1998) recognizes a band with a slightly faster mobility than *Xenopus* ATM

proteins. To determine whether X-ATM might be associated with other proteins in *Xenopus* extracts we analysed extracts by native gel electrophoresis. We detected a native, high molecular weight X-ATM-containing complex with an estimated size of 500-kDa (Figure 7). In addition, using sucrose gradient centrifugation, we detected a high molecular weight form of ATM in extracts consistent with our electrophoresis analysis (data not shown).

Discussion

Xenopus *ATM*

We report the cloning of a *Xenopus* homolog of ATM, the gene mutated in Ataxia-Telangiectasia. Although we have not yet isolated the full-length clone, sequence comparison spanning 50% of the predicted ORF, establishes clearly that we have isolated a *Xenopus* ATM homolog rather than a member of the PI3-kinase family. The amino acid levels of identity between X-ATM and human or mouse ATM are 71 and 69%, respectively while human ATM is 85% identical to mouse ATM over the same half of the protein. In comparison, there is only weak homology outside the kinase domain between X-ATM and ATR, mei41 or rad3, three genes which are forming the 'ATR/rad3' branch of this family of protein kinases (Hoekstra, 1997).

The high degree of conservation between X-ATM and ATM within the kinase domains (85% identity) strongly suggests that the catalytic activity of these two proteins will be conserved. The decreasing levels of

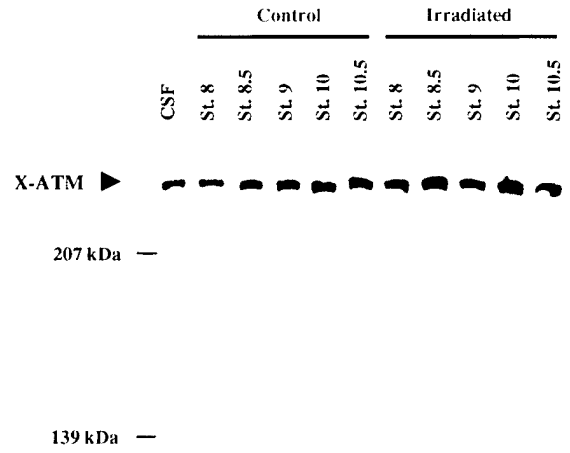

Figure 6 X-ATM protein following DNA damage. The level of ATM in extracts from control and γ-irradiated embryos between stage 8 and 10.5 was compared. Embryos were γ-irradiated (40 Gy) using a ^{60}Co source at stage 2. Embryos were collected and crushed at the indicated stages, and the soluble extract was analysed by 6% SDS–PAGE followed by Western blot analysis. ATM in a mitotic extract (CSF) is also shown

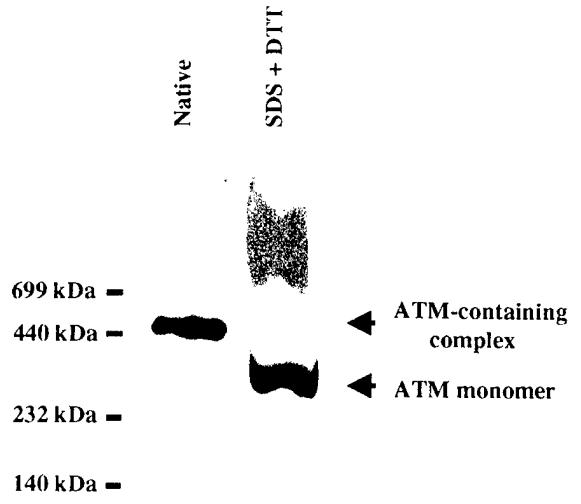

Figure 7 X-ATM complexes. Two hundred μg of egg extract were electrophoresed under native conditions and immunoblotted with X-ATM antibody (first lane). A band of approximately 450–500 kD corresponding to the complex containing X-ATM is observed as indicated. The same sample was electrophoresed in the same polyacrylamide gradient gel under reducing and denaturing conditions (second lane). The immunoreactive band migrates at 370 kDa, the molecular weight of monomeric ATM

identity outside the catalytic domains could reflect potential differences in regulation of these proteins between species.

Mutations in the ATM gene are not clustered in a specific area of the molecule or restricted to a limited number of residues, instead they span the entire ORF of the molecule. This has made it difficult to discriminate between true mutations and polymorphisms. The availability of X-ATM sequence might help resolve some of these issues by identifying mutations in conserved residues that have been originally classified as polymorphisms. Conversely, it might help confirm the location of polymorphisms in non-conserved residues.

Cell cycle patterns of X-ATM expression

We present a detailed analysis of the expression of X-ATM throughout both meiotic and mitotic cell cycles. These cell cycles are naturally synchronous in *Xenopus* and allowed the cell cycle expression of ATM to be followed in undisturbed cell cycles. We find no evidence for cell cycle oscillation in the level or the electrophoretic mobility of ATM protein during the mitotic cell cycles in cell-free extracts or during the meiotic divisions of the oocyte. This confirms previous results for human ATM obtained in cells that have been synchronized in culture (Brown *et al*., 1997; Gately *et al*., 1998). Due to the large size of X-ATM, small variations in mobility might not be detectable despite the use of very resolutive PAGE. In addition, ATM could be modified in ways that does not affect its electrophoretic mobility. Therefore we cannot rule out that X-ATM protein could be modified in a cell cycle dependent manner.

Developmental expression of X-ATM

We found that X-ATM was expressed maternally, very early during oogenesis. This might reflect the need for ATM function in growing oocytes and during meiosis. Later during embryonic development we did not observe dramatic changes in the levels of mRNA or protein which is consistent with X-ATM playing a role in all cell types throughout development. RNAse protection assays show the presence of a second protected fragment at stages 9 and 10 of smaller size than the expected protected fragment. This could be due to the two different mRNA species generated by alternative splicing. Alternatively, a stable secondary structure could exist at either end of the protected fragment giving rise to the observed pattern. Since alternative mRNA splicing has not been observed within the ORF of human ATM (Savitsky *et al*., 1995) we favor the second hypothesis.

We also followed the protein expression patterns of ATM in embryos that have been irradiated by ionizing radiation. We previously reported that such an insult induces a synchronous apoptotic program in *Xenopus* embryos, manifest at the onset of gastrulation (Anderson *et al*., 1997; Hensey and Gautier, 1997). In such embryos destined to die by apoptosis we did not observe any changes in the levels of X-ATM. Moreover, when we followed X-ATM in *Xenopus* apoptotiç extracts containing active caspase (as seen by PARP cleavage), we did not observe X-ATM cleavage despite the presence of a good consensus site for caspase cleavage (towards the C-terminal-end) of the X-ATM molecule (DIVD) (data not shown).

Localization

We clearly demonstrated that X-ATM is exclusively nuclear in the germline. *Xenopus* oocyte nuclei can be

manually isolated from their surrounding cytoplasm with virtually no cross contamination. This provides unequivocal evidence for the subcellular localization of X-ATM in this system. Although X-ATM isolated from oocytes migrated with the same mobility on PAGE as X-ATM isolated from eggs (see Figure 7), we occasionally observed a second slower migrating band on Western blots from oocytes early during vitellogenesis (data not shown).

As meiotic maturation takes place and the nuclear envelope breaks down, X-ATM becomes cytoplasmic. In the unfertilized egg, corresponding to the second meiotic metaphase, the protein is cytoplasmic as seen by its presence in cell-free extracts. The nuclear localization of the protein in oocytes might reflect the fact that X-ATM could be essential for the proper completion of meiosis as is the case in mammals (Barlow *et al*., 1998; Xu *et al*., 1996). However, during the early cleavages of the embryos, the rapid divisions lack cell cycle checkpoints and are relatively insensitive to either DNA replication inhibitors or DNA damage (Hensey and Gautier, 1997). It will be interesting to determine whether exclusion of ATM from the nucleus correlates with the lack of cell cycle checkpoints and at what time during development X-ATM becomes nuclear again.

It is interesting to compare the expression and localization of X-ATM with that of Xenopus p53 since in mammalian systems p53 has been shown to be a target and a substrate for the ATM protein kinase. *Xenopus* p53 is structurally and functionally related to human p53 (Cox *et al*., 1994; Soussi *et al*., 1987; Wang *et al*., 1995). Like X-ATM, p53 is present in oocytes and synthesized during early oogenesis (Tchang *et al*., 1993). However in contrast to what we observe for X-ATM, *Xenopus* p53 is entirely cytoplasmic in the oocyte. The difference in subcellular localization between p53 and X-ATM might provide a partial explanation for the unusually high levels of p53 protein observed in *Xenopus* oocytes and embryos.

Complex formation

We also clearly demonstrate that ATM is present in at least one larger complex than the monomeric form. The non-denaturing gels we used in these experiments allowed us to clearly resolve a complex of approximately 500 kDa, which could be disrupted following SDS treatment. It is possible that other larger complexes also exist but cannot be resolved using PAGE. We confirmed these findings using sucrose gradient centrifugation of egg extracts followed by Western blotting. We found that X-ATM was found in a high molecular weight complex sedimenting around 600 kDa (data not shown).

The finding that X-ATM is associated with other partner(s) in the simple, unregulated cell cycles of *Xenopus* cell-free extracts suggest that X-ATM might always require the association with regulatory subunits to perform its functions. ATM has been shown to interact with c-abl which is also a substrate for the kinase. It was also demonstrated that ATM interacts with β-adaptin, a protein involved in clathrin-mediated endocytosis of receptors (Lim *et al*., 1998). The identity of the *Xenopus* ATM partner(s) awaits further investigation.

Materials and methods

Cloning of Xenopus *Atm*

Degenerate RT–PCR Four degenerate primers were designed from regions of amino acid identity between the kinase domain of ATM and its yeast homologue TEL 1, with a slight bias towards the human sequence. Primers 1 and 2 were in the 5′-3′ direction. Primers 3 and 4 were in the 3′-5′ direction. The primer sequences were as follows:

Primer 1: 5′-GCGCGGATCCGA(CT)GA(CT)(CT)T(AG-CT) (AC) G (AGCT) CA (AG) GA (CT)-3′ (amino acids D-DLRQDA, positions 2719–2726 in ATM)
Primer 2: 5′-GCGCGGATCCT(AGCT)ATG(GC)A(AG)-CA (AG) GT (A GCT) TT (CT) -3′ (amino acids MQQVFQ, positions 2727–2733 in ATM)
Primer 3: 5′-GCGCATCGATA (CT) (AGCT)CC(AGCT)-A(AG)(AG)TC(ATG)AT(AG)T G-3′ (amino acids HIDLG-V, positions 2886–2892 in ATM)
Primer 4: 5′-GCGCATCGATTT(AGCT)CC(CT)TG(A-GCT)TC(AG)AA(AGCT)GC-3′ (amino acids AFEQGK, positions 2892–2898 in ATM).

Total RNA was isolated from *Xenopus* Tissue Culture (XTC) cells (RNAzol B, Tel-Test) and was subjected to a reverse transcription reaction. The cDNA product was used as a template in PCR with primers 1 and 4 in a standard PCR reaction. A fragment of approximately 480 bp was amplified, and reamplified with nested degenerate primers 2 and 4.

Library screens A λ Zap library from XTC cells was screened using the 480 bp PCR fragment as a probe and a high stringency hybridization was performed. Following three successive rounds of screening the clone XTC 3 was isolated containing a 2.1 kb insert including 600 bp of 3′ UTR.

A further 500 bp of *X-Atm* was cloned upon screening of a second λ Zap library from Stage 25 *Xenopus* embryos (a gift from Dr Hemmati-Brivanlou and Dr Harland). The TBH 4 clone was 1 kb in size and partially overlaps with the 5′ 500 bp of clone XTC 3 (see Figure 1a)

X-Atm *library construction* A Zap Express library was constructed using the Zap Express cDNA Gigapack III Gold Cloning kit, (Stratagene). Messenger RNA was isolated from XTC cell total RNA, and subjected to first strand synthesis using a primer specific for *X-Atm* (instead of the oligo DT primer provided) containing a *Xho*I site 5′-GAGAGAGACTA GTCTCGAGTGTGTCGACAAT-GTGACGTGC-3′. All cDNA's with molecular weights above 1 kb were excised, packaged into λ arms and the library titrated.

Subsequent screening of 20 000 plaques revealed a 2.5-kb clone, *X-Atm1.3* that overlaps with the 5′ 210 bp of the TBH 4 clone (Figure 1a).

5′ RACE mRNAs were isolated from XTC cells as previously mentioned and reverse transcribed. Second strand synthesis and adapter ligation was carried out using the Marathon 5′ RACE kit (Clontech). Long distance PCR was performed on the cDNA. After three rounds of PCR with nested primers an 800 bp product, clone RACE 1, was generated and resulted in a further 500 bp of new sequence (350 bp of this clone overlaps with the 5′ of clone *X-Atm1.3*, see Figure 1a).

The assembly of all preceding clones provided 5.3 kb of sequence corresponding to the 3′ UTR and 50% of the predicted ORF of the cDNA. Amino acid sequence alignments and alignment scores were performed using Geneworks software.

RNAse protection assay

The RNAse protection assay was performed according to the protocol described in the RPAII kit (Ambion). A 480-bp fragment from clone TBH 4 (nucleotide position 5–485) was used as a probe (Figure 1a). The probe was hybridized with 15 μg of XTC cell total RNA and with RNA from samples of ten *Xenopus* eggs or embryos, stages 5, 9, 10, 17 and 26. After hybridization the samples were digested with RNAse A and H, and analysed by PAGE.

Bacterial expression of X-ATM fragment and generation of X-ATM antibodies

A 500 bp fragment from *X-Atm* clone TBH 4 (positions 5–485) was amplified by the forward primer 5′-GACGTCCTTAATAATCTGATT-3′ upstream of an internal *Nco*I site and the reverse primer 5′-GCGCGGATCCGAGATTAAGGCCCCCGGCCAG-3′ which contains a *Bam*HI site (Figure 1a) and cloned into the *Nco*I and *Bam*HI sites of the His_6 Expression vector PQE60.

We also cloned the 3′ 1.5 kb of the *X-Atm* ORF into a T7 vector that allowed the production of a 65-kDa polypeptide in rabbit reticulocyte lysate (T7/TBH-XTC).

X-ATM was expressed in *E. coli* and affinity purification of the denatured peptide was carried out as described in the QIAexpress protocol (Qiagen). 2 mg of protein was run on a 15% preparative polyacrylamide gel, the band excised from the gel and used to immunize rabbits.

Sera were tested by Western blotting against the *in vitro* translated X-ATM. Sera from two rabbits gave a strong signal and the antibodies from rabbit B were further purified on an affinity column prepared by coupling the 20 kDa X-ATM protein fragment to CNBr sepharose beads.

Protein sample preparation

Cycling and CSF extract Cycling extracts were prepared from unfertilized *Xenopus* eggs, as described in (Murray, 1991).

Embryos Batches of 20 embryos from the indicated stages were collected and snap frozen in liquid nitrogen. The embryos were crushed in a modified RIPA lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 0.5% Nonidet P-40, 0.05% SDS) containing pepstatin, leupeptin, aprotinin and AEBSF (10 μg/ml each). One hundred μg of protein from each sample used in Western blots.

Oocytes Stages II, IV, and VI (Dumont, 1972) were gently crushed in 150 mM NaCl, 50 mM Tris, pH 7.4 and processed in the same way as embryos. Oocyte maturation, i.e. meiosis, was induced *in vitro* by 10 μM progesterone in MBS-H (Gautier and Maller, 1991).

Cells XTC cells were washed in ice-cold PBS, pelleted and resuspended in RIPA lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.4, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). Nuclear Protein Extracts (NPE) were prepared according to (Walter *et al*., 1998).

Western blotting

Protein samples were separated under denaturing conditions on 20 cm, high resolution 6% SDS polyacrylamide gels, and transferred to nitrocellulose. After blocking in 5% milk in PBS, membranes were incubated with either affinity purified X-ATM antibody or human ATM antibody AT 1.8 (a gift from Dr T Yen), followed by incubation with horse-radish peroxidase-conjugated secondary antibodies. Proteins were visualized using enhanced chemiluminescence (Amersham).

Quantitation of X-ATM in Xenopus *embryos*

Linear T7/TBH-XTC clone (Figure 1a) was used as a template in an *in vitro* transcription/translation reaction with 2 μl of [^{35}S]methionine of known specific activity. One μl samples from the completed reaction were counted in a scintillation counter to determine the average number of counts corresponding to the calculated number of moles of [^{35}S]Met. The amount of [^{35}S]Met incorporated into the X-ATM band was then determined by electrophoresing 1–3 μl of the translation reaction on a 10% SDS–PAGE gel and transferring to nitrocellulose. The radiolabeled band was excised and counted, and counts were converted to pmoles of [^{35}S] per μl of the translation. Western blots of both the translation products and CSF extract were carried out in parallel and the band intensities of different dilutions were compared and hence, knowing the number of methionines in the translation product, we were able to determine the molarity of X-ATM in CSF extract. This was extrapolated to embryos by comparing band intensities in Western blots containing CSF and extracts from a known number of embryos resulting in an estimation of the molar concentration of X-ATM per embryo.

Native gradient gel electrophoresis

Ten μl of interphase extract were either suspended in TBE containing 10% glycerol or a modified buffer also containing 3% SDS and 100 mM DTT. Samples in the SDS/DTT containing buffer were heated to 80°C for 5 min. Proteins were then separated on a non-denaturing linear gradient polyacrylamide gel in 1 × TBE overnight. Before transferring proteins to nitrocellulose the gel was soaked in SDS buffer (48 mM Tris, 39 mM glycine, 0.25% SDS) for 30 min at room temperature with agitation, followed by a 30 min soak at 80°C (Hendrickson *et al*., 1996). Proteins were probed with the X-ATM antibody as previously described.

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Accession number

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EXPRESSION NOTE

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Expression and subcellular localization of X-ATM during early *Xenopus* development

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Abstract *ATM*, the gene mutated in ataxia telangiectasia, is a protein essential for handling DNA strand breaks. We recently isolated the *Xenopus* homologue of *ATM*, *X-ATM* and we report here the detailed expression pattern of the protein and the mRNA during early *Xenopus* development. During the cleavage stages, ATM protein was concentrated in and around the nuclei of all cells with low levels of expression also detected in the cytoplasm. Following neurulation, increased protein levels were detected in the nuclei of developing somites and in the central nervous system. Areas of high protein expression correlated with areas of increased mRNA expression which was detected in the nuclei of somites and the developing lens.

Key words Ataxia Telangiectasia · ATM · *Xenopus* · Lens · Somites

The disease ataxia telangiectasia results from mutations of the *ATM* gene (Savitsky et al. 1995) and is characterized by a variety of clinical manifestations, including neurodegeneration, immunodeficiency, and a predisposition to cancer. At the molecular level the ATM protein regulates cellular responses to DNA strand breaks (Banin et al. 1998; Brown et al. 1997; Canman and Lim 1998). Previous studies have shown that Atm is localized to meiotic nuclei (Barlow et al. 1998) and the developing nervous system in mice (Herzog et al. 1998). *ATM* mRNA is ubiquitously expressed in mouse embryos with elevated mRNA expression levels in the cerebellum and other regions of the CNS suggesting an early developmental requirement for ATM in the nervous system (Chen and Lee 1996; Soares et al. 1998). However, little is known about ATM expression during early development. We cloned the *Xenopus* homologue of human *ATM* (Robertson et al. 1999), and using whole-mount in situ hybridization and immunohistochemistry, have analyzed the expression pattern of *X-ATM* during early development in *Xenopus* embryos.

Using an antibody that specifically recognizes XATM in immunoblotting and immunoprecipitation experiments (Robertson et al. 1999), we analyzed Atm expression by whole-mount immunohistochemistry at representative stages of *Xenopus* development (Nieuwkoop and Faber 1956). Atm is expressed in all blastoderm nuclei in a stage 8 embryo (Fig. 1 A). Sectioning embryos labeled in whole-mount revealed that Atm is predominantly nuclear and perinuclear as evidenced by the overlapping DAPI stain (Fig. 1B, C). However the staining is not homogeneous throughout all nuclei. A component of the protein is cytoplasmic and is also detected in a fiber-like network radiating from the dense area of nuclear staining through the cytoplasm (Fig. 1D). Punctate areas of stronger staining are detected along the fibers. Similar expression patterns were detected in 32-cell (stage 6) and 64-cell (stage 7) embryos and we did not observe any change in the expression pattern following γ-irradiation (data not shown). These observations are consistent with our previous observation that Atm is nuclear in both the male and female germline in *Xenopus* (Robertson et al. 1999). A number of biochemical studies have also shown that human Atm is a nuclear protein (Brown et al. 1997; Chen and Lee 1996; Gately et al. 1998).

The protein was ubiquitously expressed during gastrulation and early neurulation with the highest level of staining detected in the nuclei (data not shown). By mid to late neurulation (stage 18), differential tissue expres-

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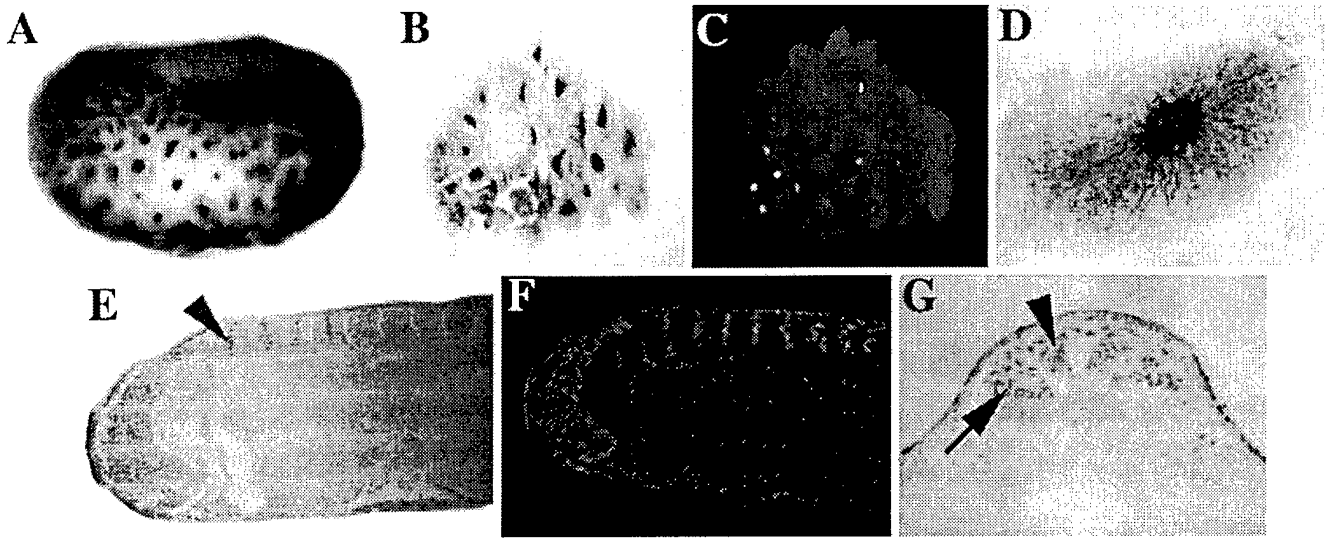

Fig. 1A–G Atm protein expression and subcellular localization in stage 8 and stage 21 embryos. Immunohistochemistry was performed using a 1:1000 dilution of affinity purified α-ATM (Robertson et al. 1999). **A** Stage 8, lateral view, whole-mount α-Atm showing strong nuclear staining. Embryos processed in a similar manner but omitting the primary antibody showed no staining. **B** Section from a whole-mount embryo. **C** The same section DAPI-stained, showing nuclear staining overlaps with ATM staining. In some cells the strong Atm staining masks the DAPI stain. **D** Higher magnification of a section showing Atm staining in a single cell. **E** Stage 21, parasagital section. The strong somite staining is due to high levels of Atm expression in the somite nuclei (*arrowhead*). **F** DAPI staining of the same section as in **E**, indicating the localization of the nuclei. **G** Stage 21, transverse section. High levels of Atm expression in both the nuclei of the somites (*arrow*) and the neural tube (*arrowhead*) are observed

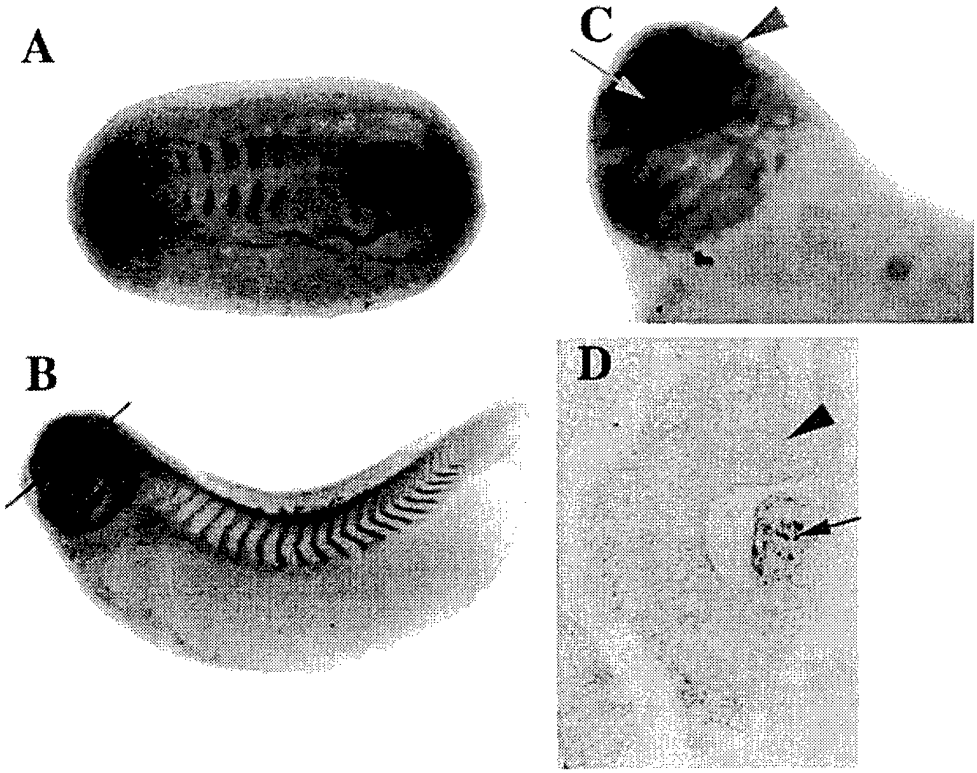

Fig. 2A–D Atm mRNA expression at stage 18 (**A**), and 32 (**B–D**). A 2.5 kb fragment of the *X-ATM* cDNA, corresponding to *X-ATM* 1.3, a region 5' of the putative kinase domain (Robertson et al. 1999), was transcribed to generate a digoxigenin labeled antisense probe. Atm mRNA expression is detected in the somites and developing CNS (anterior staining) at stage 18 (**A**). **B** Stage 32, lateral view, line indicates the section shown in **D**. High levels of mRNA are maintained in the somite nuclei and are also detected in the branchial arches, developing brain and eye. **C** Stage 32, a shorter staining time reveals more detail in the anterior staining. The lens nuclei are strongly stained (*arrow*) and specific regions of the brain show high levels of mRNA expression (*arrowhead*). **D** A transverse section at the level of the mesencephalon reveals a high level of mRNA expression in the developing lens (*arrow*) with a lower level of expression throughout the retina (*arrowhead*). Embryos processed in a similar manner but omitting the antisense probe showed no staining

sion, as evidenced by increased protein levels in the central nervous system and somites, became apparent (data not shown). As seen in Fig. 1 E and G, the nuclei of the developing somites and neural tube were most strongly stained at stage 21. Some staining of the epidermis was also observed. The protein continued to be expressed throughout the developing embryo, and by stage 31/32, elevated expression levels were maintained in the somite nuclei in addition to the developing CNS and the retina and lens of the developing eye (data not shown).

High background staining prevented the detection of specific mRNA expression patterns prior to stage 17, but beyond this stage, increased levels of mRNA expression correlated with areas of increased protein expression. Starting at mid to late neurulation, mRNA expression was detected in the developing somites (Fig. 2A, B). In the stage 18 embryo shown in Fig. 2A, mRNA is highly expresssed in the somite nuclei and this high level of expression is maintained up to stage 37/38, the period of somite differentiation. In addition to high levels of expression in the somite nuclei, a stage 32 embryo also shows high mRNA expression in the branchial arches, brain, and developing eye, particularly the lens (Fig. 2C, D).

The predominantly nuclear localization of Atm is consistent with its putative role in regulating the cellular response to DNA strand breaks. The ubiquitous expression pattern is also consistent with its function in the DNA damage response, an important cellular response. Studies in mouse have also shown increased expression of ATM in the central nervous system, pointing to an early developmental requirement for ATM in the nervous system (Chen and Lee 1996; Soares et al. 1998). The localized expression such as that detected in the developing somites and lens might reflect a physiological function involving DNA breaks. *ATM*, among other genes, is believed to be involved in DNA damage recognition (Smith et al. 1999). This role is critical in cycling cells as a component of the checkpoint pathway, but could also play a role during development and differentiation. Interestingly, mutations in several genes implicated in DNA end-joining and ligation show developmental abnormalities (Barnes et al. 1998; Tebbs et al. 1999).

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Reconstitution of an ATM-Dependent Checkpoint that Inhibits Chromosomal DNA Replication following DNA Damage

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Summary

Cell cycle checkpoints lead to the inhibition of cell cycle progression following DNA damage. A cell-free system derived from *Xenopus* eggs has been established that reconstitutes the checkpoint pathway inhibiting DNA replication initiation. DNA containing double-strand breaks inhibits replication initiation in a dose-dependent manner. Upon checkpoint activation, a prereplicative complex is assembled that contains ORC, Cdc6, Cdc7, and MCM proteins but lacks Cdc45. The checkpoint is ATM dependent. Cdk2/CyclinE acts downstream of ATM and is downregulated by Cdk2 phosphorylation on tyrosine 15. Cdk2AF/CyclinE is refractory to checkpoint signaling, and Cdc25A overrides the checkpoint and restores DNA replication. This report provides the description of a DNA damage checkpoint pathway that prevents the onset of S phase independently of the transcriptional function of p53 in a vertebrate organism.

Introduction

Cells respond to DNA damage by activating checkpoint pathways that delay progression through the cell cycle (Hensey and Gautier, 1995; Elledge, 1996). These signaling pathways operate throughout the cell cycle. Activation of a DNA damage checkpoint can induce G1, S, or G2 phase delay. These surveillance mechanisms are essential to maintain the integrity of the genome. They ensure that damaged DNA templates are neither replicated nor segregated to the daughter cells until repaired. Failure to monitor and to signal following DNA damage is a hallmark of cancer cells (Hartwell and Kastan, 1994).

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A regulatory network of proteins has been identified that participates in DNA damage checkpoint signaling. Central to this network is the ATM protein, the product of the gene mutated in the human disease ataxia-telangiectasia (Savitsky et al., 1995). Ataxia-telangiectasia (A-T) is an autosomal recessive disease that displays a complex phenotype (Boder and Sedgwick, 1970; Shiloh, 1998). Patients exhibit a progressive cerebellar ataxia, in addition to severe immune deficiencies, gonadal atrophy, telangiectases, increased risk for cancer—particularly lymphomas—and radiation sensitivity. Cell lines from A-T patients show enhanced radiosensitivity to ionizing radiation (Lavin and Shiloh, 1997), increased chromosomal loss and breakage, and abnormal telomere morphology (Smilenov et al., 1997; Vaziri et al., 1997). A-T cells are defective in cell cycle checkpoints in G1, S, and G2 (Meyn, 1995; Beamish et al., 1996; Hoekstra, 1997). The cellular phenotype of A-T suggests a defect in handling DNA breaks formed by external insults or as a result of normal physiological processes such as meiotic recombination and maturation of the immune system. ATM is a serine/threonine kinase for which structurally and functionally similar proteins have been identified and characterized in *S. cerevisiae*, *S. pombe*, *D. melanogaster*, and *X. laevis* (Weinert, 1992; Hari et al., 1995; Bentley et al., 1996; Robertson et al., 1999; Sibon et al., 1999). When exposed to ionizing radiation, mammalian $ATM^{-/-}$ cells cannot prevent S phase entry and undergo radio-resistant DNA synthesis (RDS) (Jeggo et al., 1998).

Following ionizing radiation, ATM phosphorylates and participates in the activation of p53 (Banin et al., 1998; Canman et al., 1998; Khanna et al., 1998). Activated p53 promotes the synthesis of p21, a cyclin-dependent kinase inhibitor with preferential affinity for Cdk2/CyclinE, delaying cell cycle prior to S phase entry. ATM also phosphorylates and activates the Chk1 and Chk2 protein kinases (Matsuoka et al., 1998; Chaturvedi et al., 1999; Chen et al., 1999). The activation of Chk1 is essential to prevent entry into mitosis in mammalian cells and in *Xenopus* extracts following DNA replication block (Kumagai et al., 1998a). Chk1 mediates G2 arrest by phosphorylating Cdc25 tyrosine phosphatase at a serine residue, creating a binding site for 14-3-3, thus inhibiting Cdc25 activity (Furnari et al., 1997; Sanchez et al., 1997; Zeng et al., 1998). Chk2, which is the vertebrate homolog of *S. pombe* Cds1 and *S. cerevisiae* Rad53, regulates p53 directly by phosphorylation (Chehab et al., 2000; Hirao et al., 2000; Shieh et al., 2000). In yeast, where p53 is not present, the ATM homologs Mec1/Tel1 for *S. cerevisiae* and Rad3 for *S. pombe* function through Chk1 to regulate cell cycle arrest in G2 and are also important for G1 and S phase delay (Weinert, 1992; Siede et al., 1993; Bentley et al., 1996). In all organisms, the mechanisms by which S phase entry is prevented in an ATM/Mec1/Rad3-dependent fashion are poorly understood.

In vertebrates, the molecular dissection of the G1 arrest mechanism has mainly focused on studies of p53-dependent pathways. Responses mediated by p53 require transcription and de novo protein synthesis and therefore are more appropriate for a nonacute response to DNA damage. Furthermore, in many systems DNA

damage induces p53-dependent apoptosis rather than cell cycle delay (Carr, 2000). It is therefore not surprising that p53-independent checkpoint signaling following DNA damage operates in mammalian cells at the onset of and throughout S phase (Lee et al., 1997; Larner et al., 1999; Agami and Bernards, 2000; Mailand et al., 2000).

Little is known about the molecular step(s) that must be blocked to prevent replication initiation in the presence of DNA damage. The initiation of DNA replication in eukaryotes requires the assembly of a prereplicative complex (pre-RC) on chromatin. The pre-RC is formed by the stepwise assembly of the origin recognition complex (ORC), the loading factor Cdc6, the putative replicative helicase mini chromosome maintenance proteins (MCMs), and Cdc45 (Stillman, 1996; Newlon, 1997), which subsequently allows polymerase loading (Mimura and Takisawa, 1998). Initiation also requires the concerted activity of the cell cycle–regulated protein kinases Cdk2/CyclinE, PKA, and Cdc7/Dbf4 (Strausfeld et al., 1994; Jackson et al., 1995; Yan and Newport, 1995; Costanzo et al., 1999; Roberts et al., 1999; Jares and Blow, 2000).

Cell-free systems derived from *Xenopus* eggs have been widely used to elucidate the biochemical bases of cell cycle transitions. They have been especially powerful in probing the regulation of entry into S phase and into mitosis. In such systems, G2 cell cycle checkpoint activity is monitored by the inhibition of nuclear envelope breakdown following experimental interference with DNA replication (Dasso and Newport, 1990; Kumagai et al., 1998a, 1998b; Guo and Dunphy, 2000).

We have designed a cell-free system to study the DNA damage checkpoint that prevents S phase entry. We monitored the effect of small damaged DNA templates on chromosomal DNA replication. This in vitro system reconstitutes a functional cell cycle checkpoint in which DNA containing double-strand breaks (DSBs) inhibits DNA replication in a dose-dependent manner. This system has revealed a novel checkpoint signaling pathway that operates through ATM. Cdk2/CyclinE is inhibited by phosphorylation of tyrosine 15 on the Cdk2 subunit, thus preventing the loading of Cdc45 at the origin of replication and initiation of DNA synthesis. This signaling is rapid, independent of de novo protein synthesis and, by inference, independent of p53 transcriptional activity.

Results

DNA Damage Checkpoint Inhibition of S Phase Entry in a Cell-Free System

To recapitulate cell cycle checkpoint response to DNA damage at the onset of S phase, we modified a cell-free system designed to study initiation of DNA replication (Chong et al., 1997). In this system, interphase *Xenopus* egg extracts are subjected to ultracentrifugation followed by sequential PEG precipitation (Figure 1 and Experimental Procedures). Two fractions (M and B) are obtained. Both fractions are required to initiate replication of chromosomal DNA assembled in an initiation-incompetent extract prepared in the presence of 6-dimethylaminopurine (6-DMAP) (Figure 2A). M contains MCM proteins, the putative replicative DNA helicase, whereas B is required for the loading of MCMs onto chromatin and for activation of the pre-RC (Chong et al., 1995).

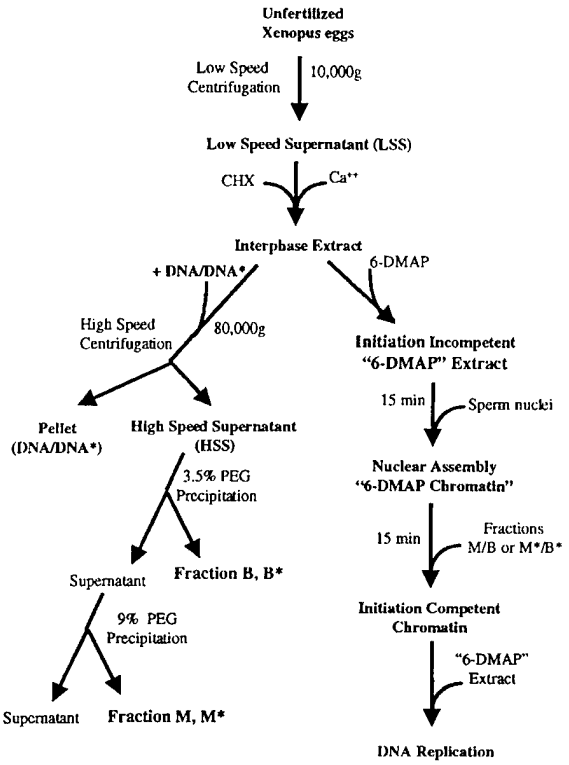

Figure 1. Fractionation Scheme and Reconstitution of an In Vitro System for the Study of the DNA Damage Checkpoint

Unfertilized *Xenopus* eggs were crushed at 10,000 × g. The resulting CSF-arrested low-speed supernatant was treated with cycloheximide (CHX) and calcium to promote exit from mitosis. This interphase extract was incubated with circular (DNA) or DSB-containing (DNA*) DNA molecules. Extracts were clarified by high-speed centrifugation. The resulting pellet containing all DNA molecules was discarded. The supernatant was precipitated with 3.5% PEG yielding fractions B and B*. The resulting supernatant was further precipitated with 9% PEG, yielding fractions M and M*. Initiation-incompetent extract ("6-DMAP" Extract) was obtained by treating a CSF-arrested extract with the kinase inhibitor 6-DMAP for 15 min and with calcium for an additional 15 min. 6-DMAP chromatin was prepared by isolating sperm nuclei assembled in 6-DMAP extract for 15 min to permit efficient unpacking of chromatin. DNA replication reactions were assembled by mixing fraction M and B or M* and B* in different combinations with 6-DMAP chromatin for 15 min, and replication was monitored in 6-DMAP extracts.

To activate the DNA damage checkpoint, the extract was incubated with DNA molecules containing DSBs. Purification of the treated extract yields two fractions corresponding to M and B, designated M* and B*, which were tested for their ability to support replication initiation.

DNA containing DSB was obtained by digesting double-strand circular plasmid with various restriction enzymes to yield blunt-ended fragments or fragments with 3′ or 5′ overhangs (Figure 2B and data not shown). Alternatively, bacteriophage λ DNA was digested with restriction enzymes to generate increasing numbers of DNA fragments (Figure 2C). Both broken and intact DNA molecules sedimented during the first ultracentrifugation step of the fractionation procedure (Figure 1) and were discarded. The M* and B* fractions were essentially free of DNA as determined by colorimetric assay and by gel electrophoresis (data not shown).

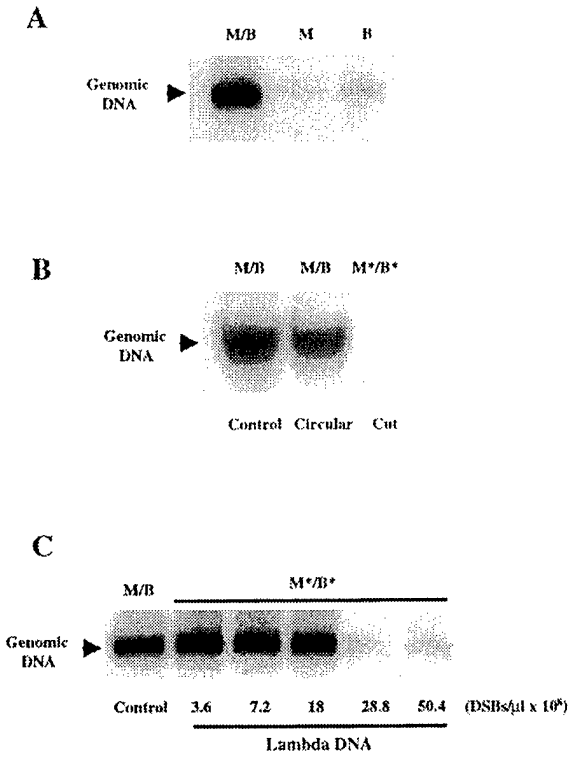

Figure 2. Inhibition of Chromosomal DNA Replication by Small DSB-Containing DNA Templates In Vitro

Genomic DNA replication was monitored by incorporation of α-^{32}P-ATP into 6-DMAP chromatin supplemented with M and B fractions.
(A) Control experiment showing replication in presence of both M and B fractions (M/B), M alone (M), or B alone (B).
(B) DNA replication in presence of fractions purified from extracts incubated with DNA. No DNA: Control (M/B); uncut plasmid: Circular (M/B); plasmid digested with HaeIII containing DSBs: Cut (M*/B*).
(C) DNA replication in presence of fractions purified from extracts containing an increasing amount of DSBs, as indicated.

To assay replication initiation, fractions M and B were incubated with chromatin isolated from an extract treated with 6-DMAP to permit the efficient unpacking of the highly condensed sperm chromatin. After incubation, the mixture was supplemented with initiation-incompetent extract to permit replication elongation. DNA replication was monitored by incorporation of α^{32}P-dATP into chromosomal DNA followed by gel electrophoresis. As previously described, fractions M and B together (M/B) supported initiation, whereas B or M alone did not (Figure 2A). In contrast, fractions M* and B* (M*/B*), obtained from extracts incubated for 15 min with fragmented plasmid DNA, were unable to promote DNA replication regardless of the type of ends (blunt, 3′ or 5′ overhangs) present in the damaged template. Treatment of extracts with intact circular DNA yielded M and B fractions with full activity (Figure 2B). Similarly, addition of DSB-containing DNA to extracts in the absence of incubation did not inhibit subsequent DNA synthesis (data not shown).

In an experiment performed with λ DNA digested to yield increasing numbers of DSBs, DNA replication was abolished in a dose-dependent manner above a threshold of 18–28 10^8 ends/μl (Figure 2C).

To determine which fraction was inactivated by fragmented DNA, we assembled the initiation reaction using M* and B (M*/B) or M and B* (M/B*). Figure 3A shows that M*/B supported DNA replication, but M/B* did not. This indicates that fraction B* is inactive in the initiation assay. The inactivity of B* might reflect the presence of an inhibitory signal. Alternatively, the fragmented DNA might titrate a factor(s) required for replication initiation. To distinguish between these possibilities, we mixed B, B*, and M fractions (M/B/B*) and monitored DNA replication. The presence of B* inhibited DNA replication more than 90% compared to a control reaction (M/B). We conclude that B* contains a dominant inhibitory signal (Figure 3B). The failure of B to complement rules out the possibility that B* is depleted for an essential factor.

X-ATM Is Required for S Phase Entry Checkpoint Signaling

We investigated whether the ATM protein kinase played a role in this checkpoint pathway. The 370 kDa *Xenopus* ATM (X-ATM) protein is expressed maternally and present in egg extracts (Robertson et al., 1999). We first traced the partitioning of X-ATM during the fractionation procedure using specific anti-X-ATM antibodies (Experimental Procedures). We found that X-ATM was present exclusively in the B fraction (Figure 3C). Interestingly, when the interphase extract was incubated with fragmented DNA prior to fractionation, some X-ATM cosedimented with the DNA during ultracentrifugation. In contrast, no X-ATM was present in the pellet obtained from an extract treated with circular plasmid (data not shown). This result strongly suggests that the checkpoint involves X-ATM bound to DNA (Figure 3C). We next used chemical inhibitors of ATM and ATM-like protein kinases to assess the role of X-ATM in the checkpoint response. Interphase extracts were incubated with fragmented DNA in the presence or absence of 5 mM caffeine or 200 nM wortmannin, two known inhibitors of ATM at these concentrations (Blasina et al., 1999; Sarkaria et al., 1999; Chan et al., 2000; Zhou et al., 2000). As seen in Figure 4A, fractions treated with inhibitors of ATM were able to support initiation of DNA replication to the same extent as controls. To demonstrate unambiguously the involvement of X-ATM, we fractionated an interphase extract that was preincubated with affinity-purified anti-X-ATM antibodies and fragmented DNA. The resulting M* and B* fractions were fully active in supporting replication initiation. We conclude that signaling induced by damaged DNA depends on X-ATM (Figure 4B).

Checkpoint Activation Inhibits Cdc45 Binding to Pre-RC

Initiation of DNA replication requires the stepwise assembly of protein complexes into pre-RC. We asked whether the checkpoint acted at one of the pre-RC assembly steps. Chromatin on which pre-RC components were assembled during incubation with M/B or M*/B* fractions was purified. The composition of this chromatin was determined by Western blotting with specific antibodies against different components of pre-RC. The binding of *Xenopus* ORC2, *Xenopus* Cdc6, *Xenopus* Cdc7, and all *Xenopus* MCM proteins to chromatin was unaffected by checkpoint activation (Figure 5). In contrast, Cdc45 was present only in chromatin assembled in M and B fractions (Figure 5). The failure of Cdc45 to

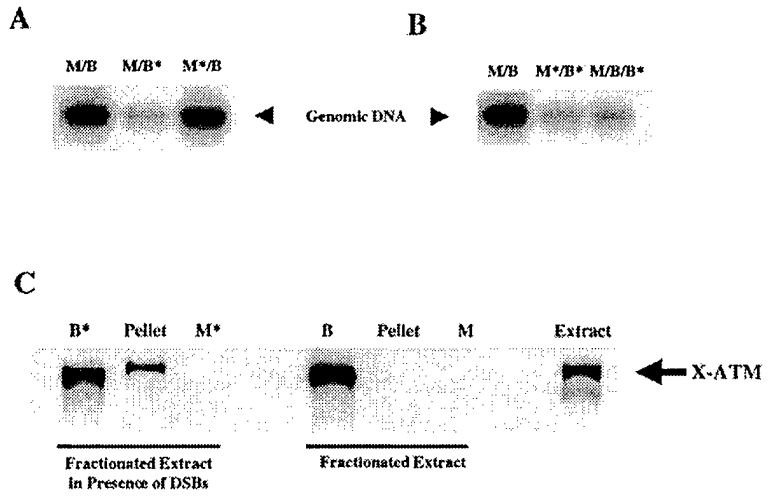

Figure 3. Fraction B* Contains X-ATM and a Dominant Signal that Inhibits DNA Replication

(A) Genomic DNA replication was monitored in extracts containing the following combinations: M/B, M/B*, and M*/B.

(B) M/B, M*/B*, and M/B/B*.

(C) X-ATM protein was traced by Western blot analysis throughout the extract fractionation procedure either in the presence of DSB-containing DNA (first three lanes) or not (next three lanes). The right lane shows an interphase extract as a control for electrophoretic mobility.

bind chromatin was X-ATM dependent, since M* and B* fractions prepared from extracts treated with anti-X-ATM antibodies supported the assembly of Cdc45 into pre-RC (Figure 5). Figure 5 also shows that the concentration of Cdc45 was identical in the M/B and M*/B* fractions (bottom panel). We therefore conclude that checkpoint activation inhibits Cdc45 assembly into pre-RC in an X-ATM-dependent fashion. Note that Cdc45 addition to pre-RC is required for loading of DNA polymerases and represents a late step in the activation of pre-RC.

Checkpoint Activation Inhibits Cdk2 Protein Kinase

The assembly of Cdc45 into pre-RC is thought to depend upon Cdk2 activity (Mimura and Takisawa, 1998; Zou and Stillman, 1998). The influence of fragmented DNA on Cdk2 kinase was assayed in the various fractions described in Figures 3 and 4 (see Experimental Procedures). The activity of Cdk2 was significantly reduced in M*/B* compared to M/B fractions (Figure 6A). The patterns of Cdk2 downregulation and DNA replication inhibition coincided precisely. Fraction B* contained a dominant inhibitor of Cdk2 activity (Figure 6A). Furthermore, Cdk2 activity was restored to control levels in B*/M* prepared from extracts treated with caffeine, wortmannin, or anti-X-ATM antibodies (Figure 6A).

Cdk2 Activity Is Inhibited by Tyrosine 15 Phosphorylation

Cyclin-dependent kinase activity is regulated by a variety of mechanisms, including association with cyclin, phosphorylation of the catalytic subunit, and binding of inhibitory proteins. We determined the mechanism by which Cdk2 was downregulated following checkpoint activation. Cdk2 is found associated with Cyclin E in interphase *Xenopus* egg extracts (Rempel et al., 1995). The total levels of both Cdk2 and Cyclin E proteins in the various fractions were unaffected by checkpoint activation (Figure 6B). In addition, the amount of Cyclin E associated with Cdk2 was equivalent in M/B and M*/B* extracts (Figure 6B, third panel). *Xenopus* extracts contain Xic1, a maternally inherited Cdk inhibitor of the p21/p27 family (Su et al., 1995; Shou and Dunphy, 1996). We measured the association of Xic1 with Cdk2 by immunoprecipitating Cdk2 and performing a Western blot analysis with anti-Xic1 antibodies. As expected for a member of the p21/p27 family of inhibitors, a fraction of Xic1 coprecipitated with Cdk2/CyclinE. However, this fraction was similar in M/B and M*/B*. Thus, the checkpoint does not inhibit Cdk2 by enhancing Xic1 binding (Figure 6B, fourth panel). Finally, Cdk2 downregulation correlates with phosphorylation of tyrosine 15. Using an antibody that recognizes specifically the tyrosine 15–phosphorylated epitope, we determined that Cdk2 in M*/B* was more phosphorylated than the Cdk2 in control extracts (Figure 6B, bottom panel).

We determined whether the inhibition of Cdk2/CyclinE activity and of DNA replication was due to tyrosine 15 phosphorylation. We reasoned that if tyrosine 15 phosphorylation was the major mechanism by which Cdk2 kinase activity was inhibited, a nonphosphorylatable form of Cdk2 at the inhibitory sites (Cdk2AF) should be refractory to checkpoint signaling. We added recombinant Cdk2WT/CyclinE (wild-type Cdk2) or Cdk2AF/CyclinE (a nonphosphorylatable Cdk2) to M*/B* frac-

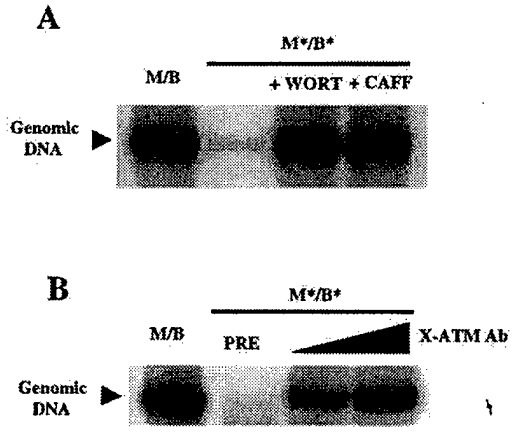

Figure 4. Inhibition of DNA Replication by DSBs Is Sensitive to Wortmannin and Caffeine and Is Dependent on X-ATM

DNA replication was monitored using fractions derived from control extract (M/B) or extracts treated with DSB-containing DNA (M*/B*).

(A) Fractions M*/B* were prepared from extracts containing no drug (lane 2), 200 nM wortmannin (lane 3), or 5 mM caffeine (lane 4).

(B) Fractions M*/B* were prepared from extracts incubated with preimmune serum (lane 2) or increasing amounts of affinity-purified antibodies against X-ATM (lanes 3 and 4).

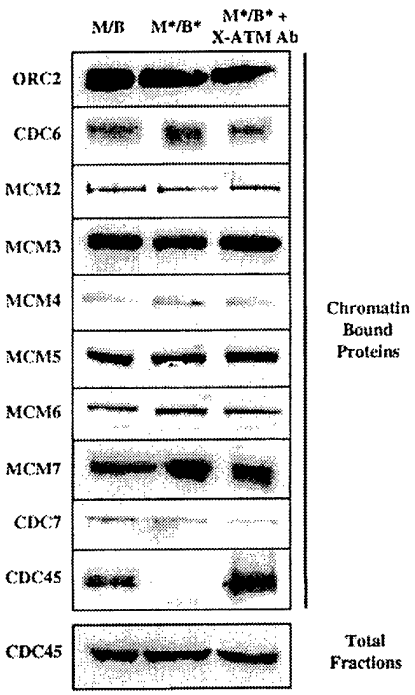

Figure 5. Consequences of Activation of the DNA Damage Checkpoint on the Pre-RC Assembly

Chromosomal DNA was purified following incubation with fractions M/B (left lane), M*/B* (center lane), or M*/B* derived from an extract incubated with anti-X-ATM antibodies (right lane). The chromatin was subjected to PAGE followed by Western blotting with specific antibodies against the pre-RC components as indicated on the left of the panels. The levels of Cdc45 protein in whole extract are shown in the bottom panel.

tions. We added identical amounts of Cdk2 kinase activity (WT or AF) corresponding to twice the endogenous Cdk2 kinase level. In these conditions, Cdk2AF/CyclinE was refractory to checkpoint signaling and restored DNA replication to M/B levels, while Cdk2WT/CyclinE did not restore DNA replication (Figure 6C). When added in a 10-fold excess over the endogenous levels, recombinant Cdk2WT/CyclinE was able to overcome the inhibition due to checkpoint signaling and restored DNA replication (data not shown). In these conditions, the replication competence of the reconstituted extract correlated with the binding of Cdc45 to chromatin (Figure 6E).

Next we measured the ability of Cdc25 to reverse the inhibitory effect of the checkpoint and restore DNA replication. We added recombinant *Xenopus* Cdc25A/WT (wild type), Cdc25A/C432A (a catalytically inactive Cdc25A), or Cdc25C/WT to M*/B* fractions (Izumi et al., 1992; Kim et al., 1999). Wild-type Cdc25A, but neither inactive Cdc25A/C432A nor Cdc25C/WT, was able to abrogate the checkpoint and restore DNA replication (Figure 6D). The end point of the DNA damage checkpoint pathway thus appears to be the phosphorylation and inactivation of Cdk2 kinase.

Discussion

Reconstitution of a DNA Damage Checkpoint In Vitro

Initiation and elongation of DNA replication are complex, multistep processes that require the assembly and the activity of a large number of proteins. In the *Xenopus* egg cell-free system described above, DNA damage activates a checkpoint that prevents S phase entry. Although titration of replication factor(s) by fragmented DNA has been reported in other systems (Wang et al., 1999), we find instead that a dominant factor inhibits DNA replication initiation in our extracts. First, the fragmented DNA that elicits checkpoint signaling in the "sensing" extract is precipitated by ultracentrifugation and is absent from the replication extract. Second, the replication extract contains all the proteins required for replication and has not been exposed to fragmented DNA. Third, the B* fraction that contains the signaling component is dominant to B; a reaction mixture containing B/B*/M fails to support replication. Fourth, signal generation requires incubation of the extract with fragmented DNA. Fifth, the inhibitory factor is absent in B* prepared in the presence of caffeine, wortmannin, or X-ATM antibodies. These observations argue against titration of an essential factor by fragmented DNA. Instead, our cell-free system represents a true ATM-dependent checkpoint pathway and reports the reconstitution of a DNA damage checkpoint in vitro.

Interestingly, the continuous presence of damaged DNA is not required for sustaining checkpoint signaling. This supports the idea that the dominant modification induced in B* by DNA damage is stable throughout the fractionation procedure and replication assay.

It is not known whether *Xenopus* early embryos undergo S phase delay in vivo in response to DNA damage. *Xenopus* embryos undergo timely cytokinesis and cortical contraction waves following γ irradiation (Hensey and Gautier, 1997). However, DNA replication was not monitored in these experiments and, since inhibition of DNA replication does not prevent mitosis and cytokinesis in *Xenopus* (Kimelman et al., 1987), it is possible that a DNA damage checkpoint operates at the onset of S phase in these embryos.

The Checkpoint Signal

To induce the checkpoint signal, we used small double-stranded DNA templates generated by restriction endonuclease digestion of either plasmid or λ DNA. At a constant mass of 10 ng/μl, the checkpoint was activated at a threshold of 18–28 10^8 ends/μl. In budding yeast, a single DSB generated in an artificial chromosome can initiate the DNA damage checkpoint response. It is proposed that the checkpoint signal in yeast is triggered by single-stranded DNA generated by exonucleolytic degradation at the site of the DSB (Lee et al., 1998). We did not detect changes in the concentration of DSB-containing DNA following signaling in the extract as measured by colorimetric assay (data not shown). Using gel electrophoresis, we did not observed detectable changes in the sizes of the DNA fragments following signaling (data not shown). Therefore, we believe that activation of the checkpoint pathway does not involve extensive processing of the DSBs. However, we cannot rule out that small modifications, such as limited processing of the ends, are taking place. Nonetheless, our results establish that DSBs are the signal that elicits the checkpoint response rather than other types of DNA lesions or free radical molecules produced following exposure of cells to ionizing radiation. Our observation that a fraction of the X-ATM cosediments with fragmented but not with intact DNA is consistent with the idea that ATM bound to DNA ends may induce the checkpoint pathway. Purified ATM has been shown to

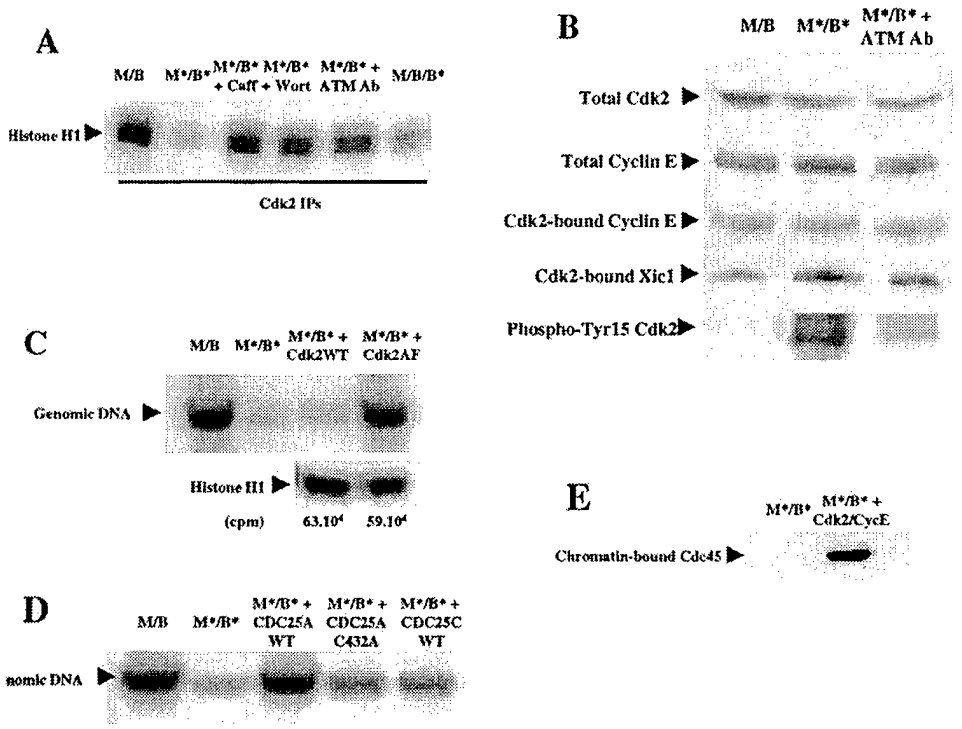

Figure 6. Activation of the Checkpoint Leads to the Downregulation of Cdk2/CyclinE and Phosphorylation of Cdk2 on Tyrosine 15

(A) Cdk2 protein kinase activity was measured using histone H1 as substrate following immunoprecipitation with an antibody specific for *Xenopus* Cdk2. As indicated, the following combinations of fractions were assayed: M/B, M*/B*, M*/B* + 5 mM caffeine, M*/B* + 200 nM wortmannin, M*/B* + ATM Ab, and M/B/B*.

(B) The levels of proteins were monitored by Western blotting in the fractions M/B, M*/B*, or M*/B* + ATM Ab as indicated above each lane. Total fractions were probed with antibodies specific against Cdk2 (top panel) and Cyclin E (second panel). Cdk2 immunoprecipitates were probed with Cyclin E antibodies (third panel), with Xic1 antibodies (fourth panel), or with phospho-tyr15-specific antibodies (bottom panel).

(C) Genomic DNA replication was monitored by incorporation of α-^{32}P-ATP into 6-DMAP chromatin supplemented with M and B fractions (M/B), M* and B* (M*/B*), M* and B* in the presence of recombinant Cdk2WT/CyclinE (M*/B* + Cdk2WT), or Cdk2AF/CyclinE (M*/B* + Cdk2AF) complexes at levels equivalent to 2-fold the endogenous Cdk2 kinase of M/B. The lower panel shows that identical amounts of Cdk2 kinase activity were added to M*/B* fractions.

(D) Genomic DNA replication was monitored by incorporation of α-^{32}P-ATP into 6-DMAP chromatin supplemented with M and B fractions (M/B), M* and B* (M*/B*), M* and B* in the presence of recombinant wild-type Cdc25A (M*/B* + Cdc25A/WT), catalytically inactive Cdc25A (M*/B* + Cdc25A/C432A), or wild-type Cdc25C (M*/B* + Cdc25C/WT).

(E) The binding of Cdc45 to the chromatin was followed by Western blotting of purified chromatin incubated in M* and B* fractions (M*/B*) or in M* and B* fractions treated with 10-fold excess of recombinant wild-type Cdk2/CyclinE complexes (M*/B* + Cdk2/CycE).

have affinity for DNA ends (Smith et al., 1999). We propose that, upon addition of DSB-containing DNA, a fraction of ATM is activated and subsequently cosediments with DNA during the fractionation procedure. ATM stably activates downstream effectors that can signal even in the absence of the checkpoint signal. This might explain why B* is dominant over B and why ATM antibodies cannot abrogate the checkpoint when added to B* after the signaling has been initiated (data not shown). Studies are in progress to analyze the DNA structures and modifications that activate the DNA damage checkpoint and to characterize the responsible protein–DNA interactions.

The Checkpoint Response Is Rapid

Signaling following DNA damage must act rapidly to prevent the replication of damaged templates at the onset of S phase. The signal must also persist to allow for repair of the damage to be coordinated with cell cycle progression. In vertebrates, a major DNA damage response that prevents S phase entry is the p53-dependent pathway. Activation of p53 induces apoptosis or p21-mediated cell cycle delay. Cell cycle response to p53 requires transcription and de novo protein synthesis of p21, in addition to a cascade of protein modifications, including ATM/ATR and Chk2 protein kinases. The p53 pathway takes several hours to induce cell cycle arrest.

We have described a DNA damage checkpoint in an extract from a vertebrate organism that does not require the transcriptional function of p53. First, transcription in *Xenopus* embryos cannot be detected until after the midblastula transition and, second, the cell-free system we describe contains cycloheximide and cannot support protein synthesis. *Xenopus* eggs and extracts do contain p53; however, we cannot exclude a nontranscriptional function for p53.

The signaling pathway described above prevents S phase entry in 15 min. This represents the time necessary for fragmented DNA to induce inhibition of Cdk2/CyclinE. Thus, this checkpoint entails only posttranslational protein modification and is, in principle, readily

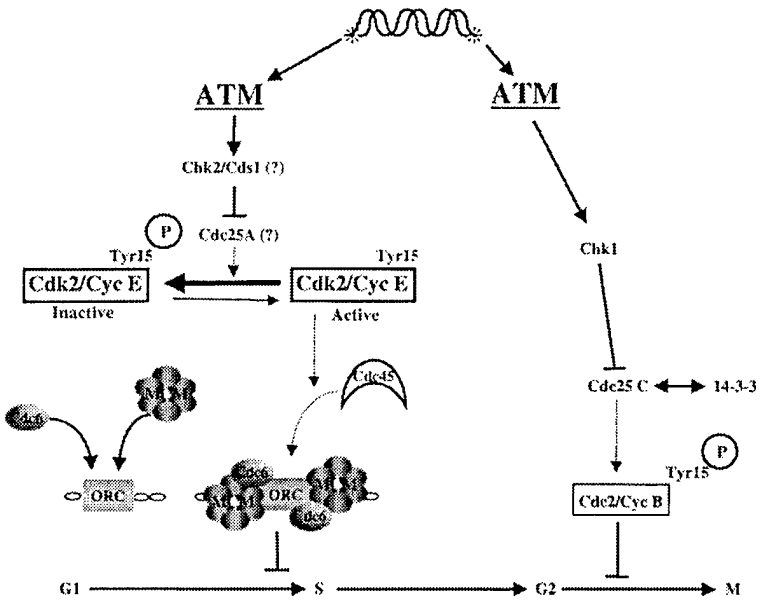

Figure 7. A Model for the ATM-Dependent Inhibition of DNA Replication Initiation following DNA Damage

Following the detection of double-strand breaks in G1, S, or G2 phases, ATM protein is activated. Prior to S phase, activation of ATM promotes a cascade of events leading ultimately to the inhibition of Cdk2/CyclinE kinase activity. We propose that ATM could activate a Chk protein (possibly Chk2/Cdcs1/Rad53), which in turn would inhibit the activity of Cdc25A. This would lead to the accumulation of inactive Cdk2/CyclinE complexes in which Cdk2 is phosphorylated on tyrosine 15. The inhibition of Cdk2 activity prevents the loading of Cdc45 on the chromatin, an essential late step in the assembly of the pre-RC.

reversible. DNA damage checkpoints at other cell cycle stages with similar characteristics have been described. These inactivate cyclin-dependent kinases by phosphorylation. A checkpoint that modifies the tyrosine 15 of Cdc2 operates at the G2/M border (Furnari et al., 1997; Sanchez et al., 1997). A second checkpoint induced by UV irradiation functions in G1 and results in the phosphorylation of tyrosine 17 of Cdk4 (Terada et al., 1995).

A Conserved DNA Damage Checkpoint

DNA damage checkpoints that prevent DNA replication in a p53-independent manner have been described in a variety of organisms. Recall that budding yeast does not contain a p53 homolog but is subject to S phase arrest by DNA damage. Rad9 may play a role in this response (Siede et al., 1993). DNA damage in mammalian cells inhibits S phase entry or ongoing replication in a p53-independent manner. Inhibition of S phase entry in CHO $p53^{-/-}$ cells takes place within 2 hr following DNA damage and coincides with 30% inhibition of Cdk2/CyclinE activity (Lee et al., 1997; Xie et al., 1998). Similar to our findings, it was recently reported that UV irradiation induces downregulation of Cdc25, leading to the accumulation of inactive Cdk2/CyclinE phosphorylated on tyrosine 15 (Mailand et al., 2000). Finally, the RDS phenotype we reconstituted in vitro is not only reminiscent of the ATM cellular phenotype but also of that of ATLD (ataxia-telangiectasia-like disease) phenotype. ATLD cells harbor mutations in the *hMRE11* gene but display normal p53 activation (Stewart et al., 1999). Taken together, these observations suggest that a checkpoint similar to the one we describe in *Xenopus* cell-free extracts may operate in mammalian cells.

Inhibition of X-ATM was sufficient to abrogate the checkpoint and to restore DNA replication to levels similar to controls. This suggests that ATM is the major protein operating in this pathway and allows us to rule out possible redundancy between ATM and ATR in the response to DSB-containing DNA. We cannot rule out, however, that ATR might function in a linear path with ATM or might operate in other signaling pathways activated by different types of damages.

Although we have shown that ATM lies upstream of Cdk2/CyclinE in the same linear pathway, we do not believe that ATM directly regulates Cdk2 activity. By analogy with the DNA damage checkpoint that inactivates Cdc2/CyclinB at the onset of mitosis, we propose that ATM might act through Chk2 kinase and Cdc25A (Figure 7), since DSBs have been shown to promote phosphorylation of Chk2 (Guo and Dunphy, 2000). Clarification of these signaling steps awaits further investigation.

Inhibition of DNA Replication

We have analyzed the effect of fragmented DNA on pre-RC assembly. The assembly of ORC, Cdc6, the MCM proteins, and Cdc7 on chromatin was resistant to checkpoint control. Activation of pre-RC requires the activity of Cdk2, Cdc7, and PKA protein kinases. Cdc7 is a cell cycle–regulated protein kinase that is essential for S phase entry in *Xenopus* and other organisms (Sclafani and Jackson, 1994; Jiang et al., 1999; Roberts et al., 1999). Cdc7/Dbf4 protein kinase is the target for a DNA replication checkpoint following exposure to hydroxyurea in budding yeast (Weinreich and Stillman, 1999). We did not observe alterations in the binding of Cdc7 to chromatin following activation of the DNA damage checkpoint. In addition, we have shown that the replication defect associated with DNA damage checkpoint activation could be fully rescued by addition of Cdk2/CyclinE protein kinase.

We showed that downregulation of Cdk2/CyclinE by phosphorylation on tyrosine 15 was necessary to prevent DNA replication following DNA damage. First, changes in the levels of endogenous Cdk2 protein kinase activity followed exactly the changes in levels of DNA replication in all experimental conditions tested. Second, 2-fold overexpression of recombinant Cdk2AF/CyclinE, but not Cdk2WT/CyclinE, could rescue the ability of an extract with an activated checkpoint to undergo DNA replication. Third, addition of recombinant Cdc25A

could also rescue DNA replication in similar conditions. Ten-fold overexpression of exogenous Cdk2WT/CyclinE in B*/M* rescued DNA replication after activation of the checkpoint by restoring Cdk2 kinase to levels comparable to B/M. The overexpression of purified Cdk2/CyclinE complex allowed the inhibitory signal contained in fraction B* to be overcome, restoring the activity to the level of B/M. Interestingly, addition of Cdk2/CyclinE (WT or AF) was only able to rescue DNA replication when added following the assembly of the pre-RC on the chromatin. Addition of Cdk2/CyclinE prior to pre-RC assembly did not rescue replication probably due to the inhibitory effect of Cdk2 on pre-RC assembly (Hua et al., 1997).

We showed that inhibition of Cdk2/CyclinE was accompanied by the inability of Cdc45 to bind to the chromatin (Figure 7). This is consistent with the observation that Cdc45 fails to bind to chromatin in extracts depleted of Cdc2 and Cdk2 by $p13^{suc1}$ beads (Mimura and Takisawa, 1998). Loading of Cdc45 on the chromatin is the last initiation event prior to the loading of the DNA polymerases. Thus, inhibiting initiation at this step provides an economical way to block DNA replication without disassembling the pre-RC.

Experimental Procedures

Cell-Free Extracts

Extract Preparation

CSF-arrested extracts were freshly prepared according to Murray (1991). For replication assays, extracts were supplemented with 100 μg/μl of cycloheximide and released in interphase with 0.4 mM $CaCl_2$. 6-DMAP (Sigma) extract was prepared in a similar way, except that 3 mM 6-DMAP was added prior to $CaCl_2$ addition.

Checkpoint Extracts

Uncut plasmid DNA, digested plasmid, or λ phage DNA were incubated in interphase extracts for 15 min to activate the checkpoint. For the rescue experiment, extracts were pretreated with 5 mM caffeine, 200 nM wortmannin, or affinity-purified anti-X-ATM antibodies (Robertson et al., 1999) following calcium addition and then incubated with damaged DNA.

Extract Fractionation

Fractions were prepared as originally described by Chong et al. (1997) to study DNA replication initiation. Interphase extracts treated as described above were subjected to fractionation to yield fractions M and B. Briefly, extracts were diluted 4-fold with cold LFB buffer (50 mM KCl, 40 mM HEPES KOH [pH 8.0], 20 mM K_2HPO_4/KH_2PO_4 [pH 8.0], 2 mM DTT, 2 mM $MgCl_2$, 1 mM EGTA, 10% sucrose, 1 μg/μl of aprotitin, pepstatin, and leupeptin). They were subjected to an ultracentrifugation step at 80,000 × g for 40 min at 4°C, using a swing out rotor. Supernatants were carefully transferred to avoid pellet contamination and supplemented with 0.075 vol of a 50% PEG solution to give a final concentration of 3.5%. Samples were incubated on ice for 30 min and spun for 10 min at 10,000 rpm at 4°C. Pellets were resuspended in LFB supplemented with 2.5 mM Mg-ATP at 5× concentration with respect to undiluted extract. This pellet corresponded to fraction B. The corresponding supernatant was adjusted to 9% PEG, and proteins were precipitated as before to yield fraction M.

DNA Templates

Chromatin Templates for Replication

Demembranated *Xenopus* sperm nuclei were prepared as described (Murray, 1991) and frozen in aliquots in liquid nitrogen. 6-DMAP chromatin was assembled as follows: 40,000 nuclei/μl. were incubated for 20 min at 23°C in 6-DMAP extract. The extract was then diluted 10-fold in chromatin isolation buffer (50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 50 mM HEPES, 0.5 mM spermine 3HCl, 0.15 mM spermidine 4HCl, 1 μg/μl aprotinin, pepstatin, leupeptin, and 0.01% Triton X-100), underlayered with the same buffer containing 30% sucrose. Chromatin was then pelleted at 6000 × g for 15 min at 4°C and was resuspended in chromatin isolation buffer supplemented with 2.5 mM Mg-ATP.

DSB-Containing Templates

To prepare molecules containing double-strand breaks, we used circular pBR 322 plasmid that was digested with restriction endonucleases to yield different types of ends (3′-, 5′-, and blunt). Since no difference was noticed with different ends, HaeIII digestion was used to provide a high number of ends. To obtain increasing number of ends, λ phage DNA was digested with XbaI, HindIII, NcoI, BstE, or HaeIII.

Replication Assays

Replication assays were performed by mixing 0.5 μl of 6-DMAP chromatin (10,000 nuclei/μl) with 1 μl of each M and B or M* and B* fraction obtained from extract treated with different types of DNA molecules. The reaction was incubated for 15 min at 23°C. Ten microliters of 6-DMAP extract was then added, and DNA synthesis was monitored by the incorporation of α-P^{32}-dATP for 90 min at 23°C following agarose gel electrophoresis (Costanzo et al., 1999). For Cdk2/CyclinE rescue experiments, fractions B* and M* were incubated for 30 min with 6-DMAP chromatin, and recombinant Cdk2/CyclinE (Hendrickson et al., 1996) was added to the reaction at 20 min.

Antibodies and Protein Assays

Chromatin Binding Assays

For chromatin binding assays, replication reactions were assembled as above, except they were scaled up 10-fold. Ten microliters of M and B fractions were incubated for 15 min with 5 μl of 6-DMAP chromatin (10,000 nuclei/μl). Following incubation, each reaction was diluted in 200 μl of chromatin isolation buffer supplemented with 0.1% Triton X-100 and underlayered with the same buffer containing 30% sucrose. The chromatin was pelleted at 6000 × g for 15 min at 4°C. The pellet was resuspended in Laemmli loading buffer. The samples were run on 10% SDS–PAGE and analyzed by Western blotting with ORC2, CDC6, MCMs, CDC7, and CDC45 polyclonal rabbit antibodies. Antibodies against ORC2, MCM3, MCM5, MCM7, and Cdc6 were a generous gift from Dr. Laskey, Dr. Madine, and Dr. Romanowski and were used as previously described (Madine et al., 1995; Hendrickson et al., 1996; Romanowski et al., 1996; Sible et al., 1998). Anti-Cdc7 was used as described previously (Roberts et al., 1999). Antibodies against XMCM4 were described previously (Hendrickson et al., 1996). Antibodies against MCM2 and MCM6 were generated against the full-length recombinant XMCM2 and XMCM6 expressed in baculovirus-infected cells. Antibodies against XCdc45 were a generous gift from Dr. Takisawa.

Cdk2 Kinase Assays

Immunoprecipitation of Cdk2 kinase activity was performed as follows: 10 μl of M and B fractions or M* and B* was incubated with 5 μl of 6-DMAP chromatin for 15 min at 23°C. The reactions were diluted in PBS supplemented with protease inhibitors and 0.2% Triton X-100 buffer to a final volume of 250 μl. The samples were precleared with protein A–Sepharose, incubated with affinity-purified rabbit Cdk2 antibody on ice for 2 hr, mixed with 25 μl of 50% protein A–Sepharose for 1 hr, washed with low and high salt buffers, and washed with EB buffer. Immunoprecipitates were incubated with EB buffer supplemented with 0.5 mg/ml histone H1 and 50 μM ATP and 1 μl of γ-P^{32}-ATP, 10 mCi/ml (>3000mC/μl). Samples were incubated at 25°C for 20 min and reaction stopped by the addition of 25 μl of 2× Laemmli buffer. The reactions then were boiled and electrophoresed on 12.5% SDS–PAGE.

Western Blot Analysis

Samples from M/B and M*/B* replication reactions were diluted in loading buffer, boiled for 3 min, electrophoresed, transferred to nitrocellulose, and probed with polyclonal antibodies specific for *Xenopus* CycE (Rempel et al., 1995), Xic1 (Su et al., 1995), and Cdk2 (Gabrielli et al., 1992). Immunoprecipitates with anti-XCdk2 were blotted with CycE antibodies and human Cdk2 antiphospho-Tyr-15 (New England Biolabs).

Cdk2 antibodies were raised against the C-terminal 16–amino acid of *Xenopus* Cdk2 (Gabrielli et al., 1992). Antibodies against Cyclin E and Xic1 were a generous gift from Dr. J. Maller.

Production of Recombinant CDK2 AF Mutant

Cdk2AF mutant was generated by PCR using a 59-mer 5′ mutagenic primer complementary to the 5′ region of hCdk2 ORF containing

base subsitutions replacing threonine 14 by alanine, and tyrosine 15 by phenylalanine and an EcoRI restriction site. The 3′ primer contained an XhoI site: 5′-GCGCGAATTCATGGAGAACTTCCAA AAGGTGGAAAAGATCGGAGAGGGCGCGTTCGGAG; 3′-GCGCCT CGAGTCAGAGTCGAAGATGGGGTACTGGC.

The PCR product was sequenced and subcloned into *pFastBac1* (GIBCO) using the EcoRI and XhoI sites. Baculovirus expression system BacToBac (GIBCO) was then used to generate viral genomic DNA encoding for Cdk2AF. Sf9 cells were transfected with viral genomic DNA carrying Cdk2AF, and viruses were harvested from the medium and used for subsequent infections.

Expression and Purification of Proteins from Insect Cells

Active Cdk2AF/CyclinE and Cdk2/CyclinE complexes were purified from Sf9 cells according to Harper et al. (1995). Briefly, 150 ml of Sf9 cells (1×10^6 cells/ml) were infected with Cdk2AF virus or Cdk2WT virus along with GST-CyclinE virus. After 48 hr, cells were harvested and lysed in buffer containing 20mM Tris-HCL (pH 8.0), 2 mM EDTA, 100 mM NaCl, 5 mM NaF, 30 mM p-nitrophenylphosphate, 1 mM PMSF, 0.5% Nonidet P-40 (Harper et al., 1995). The lysate was diluted 4-fold with 20 mM HEPES (pH 7.5), 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 1 mM ATP, and protease/phosphatase inhibitors were incubated with 1 ml of glutathione–Sepharose (Pharmacia) for 60 min. The resin was extensively washed, and GST-CyclinE/Cdk2WT or GST-CyclinE/Cdk2AF was eluted with 2 ml of 50mM Tris-HCL (pH 8.0) containing 20 mM glutathione, 120 mM NaCl plus protease and phosphatase inhibitors.

Xenopus Cdc25A, Cdc25A catalytic inactive mutant (C432A), and Cdc25C were purified according to Izumi and Maller (1993). Cdc25-expressing baculoviruses were a gift from Dr. J. Maller.

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BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Photocopy this page or follow this format for each person.

NAME	POSITION TITLE
GAUTIER, Jean	Assistant Professor, Genetics and Development / Dermatology

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Lycée Pierre de Fermat	Baccalaureat	1974	Maths, Physics
Toulouse University, France	Master	1978	Biology
Toulouse University, France	Ph.D.	1983	Developmental Biology
Toulouse University, France	Dr.Sc.	1988	Developmental Biology
University of Colorado Health Sciences Center	Post-doctoral	1988-1989	Cell Cycle
University of California San Francisco	Post-doctoral	1989-1992	Cell Cycle

RESEARCH AND PROFESSIONAL EXPERIENCE

10/1979-12/1980: School of Agronomy, Mostaganem (Algeria).Teacher (Biology, Genetics).

10/1984-09/1992: Centre National de la Recherche Scientifique, France. Permanent research scientist position as "Chargé de Recherches I".

01/1987-08/1987: Department of Biology, Indiana University (Prof. G.M. Malacinski). Visiting Scientist.

02/1988-12/1989: Department of Pharmacology, University of Colorado Health Sciences Center (Prof. James. Maller). Postdoctoral Fellow.

03/1989-06/1989: EMBO short-term fellowship, University of Oxford (Prof. P. Nurse).

12/1989-06/1992: Department of Biochemistry and Biophysics, University of California San Francisco (Prof. Marc W. Kirschner). Postdoctoral fellow. American Cancer Society Senior Postdoctoral Fellow.

06/1992-07/1993: URA 671 CNRS, Station Zoologique, Villefranche sur mer. Directeur de Recherche, CNRS (Centre National de la Recherche Scientifique).

08/1993-08/1995: Roche Institute of Molecular Biology, Associate Member.

09/1995-present: Columbia university, Department of Genetics and Development and Department of Dermatology, Assistant Professor.

HONORS AND AWARDS:

1989: Bronze Medal of the CNRS.

10/1996-09/1997: Mallinckrodt Foundation Award.

01/1997-12/2001: Irma T. Hirschl Scholar Award.

07/1997-06/2001: US Army Breast Cancer Research Program. Career Development Award.

RESEARCH PROJECTS ONGOING OR COMPLETED DURING THE LAST 3 YEARS

"The Functional Role of the Ataxia-telangiectasia Gene". To clone, characterize and study the biochemical role of the ATM protein in G2 checkpoints regulation using Xenopus egg extracts and embryos.
Principal Investigator: Jean Gautier, Ph.D.
Agency: Mallinckrodt Foundation. Period: 10/1/96 - 9/30/99.

"Skin Cancer: UVB-induced cell cycle alterations". To study the UV-induced G2 cell cycle checkpoints in keratinocytes.
Principal Investigator: David Bickers, M.D.
Co-Principal Investigator: Jean Gautier, Ph.D.
Agency: NIAMSD. RO1. Period: 10/01/98 - 09/30/01

"Cell Cycle Regulation of DNA Replication". To study the modulation of the assembly and of the function of the pre-replicative complex by cell cycle regulated protein kinases. To design in vitro systems to reconstitute chromosomal DNA replication in vitro.
Principal Investigator: Jean Gautier, Ph.D.
Agency: American Cancer Society. Period: 01/01/99 - 31/12/01.

"Cell Cycle Control by the cAMP-PKA Signal Transduction Pathway". To study the role of the cAMP-PKA pathway in regulating DNA replication and in coupling mitosis to DNA replication using Xenopus extracts.
Principal Investigator: Jean Gautier, Ph.D.
Co-Principal Investigator: Max Gottesman, M.D.
Agency: NIGMS. 1 RO1 GM56781-01A2. Period: 05/01/99 - 04/30/03.

PUBLICATIONS (Selected)

Costanzo, V., Robertson, R., Ying, C., Kim, K., Avvedimento, E., Gottesman, M., Grieco, D., and **Gautier, J.** (2000) Reconstitution of an ATM-dependent checkpoint that inhibits chromosomal DNA replication following DNA damage. *Molecular Cell*, In press.

Athar, M., Kim, A., Ahmad, N., Mukhtar, H., **Gautier, J.** and Bickers, D. Mechanism of ultraviolet B-induced cell cycle arrest in G2/M phase in immortalized skin keratinocytes with defective p53. Biochem. Biophys. Res. Comm. In press.

Shechter D., Ying C. and **Gautier J.** Methanobacterium thermoautotrophicum MCM protein is a DNA helicase.*J. Biol. Chem.* **275**, 15049-15059.

Hensey C., Robertson K. and **Gautier J.** Expression and Subcellular localization of X-ATM During Early Xenopus Development. (2000) *Development, Genes and Evolution.*. **210**, 467-469.

Romanowski P., Marr J., Madine M.A., Rowles A., Blow J.J., **Gautier J.**, and Laskey R.A.(2000). Interaction of Xenopus Cdc2·Cyclin A1 with the Origin Recognition Complex. *J. Biol. Chem.* **275**, 4239-4243.

Bao J., Talmage D., Role L. and **Gautier J.** (2000). Regulation of Neurogenesis by Interactions Between HEN1 and Neuronal LMO Proteins. *Development.***127**, 425-435.

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Costanzo V., Avvedimento E., Gottesman M., **Gautier J.** and Grieco D. (1999). PKA is Required for Chromosomal DNA replication. *Curr. Biol.* **9**, 903-906.

Hensey C. and **Gautier J.** (1999). Developmental regulation of induced and programmed cell death in Xenopus embryos. *Ann. N.Y. Acad. Sci.* (In press).

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Hensey C. and **Gautier J**.(1995). Regulation of cell cycle progression following DNA damage "Progress in Cell Cycle Research". Vol **1**, 149-162 Plenum Press.

Gautier J. (1993) New style or new journal? (Journal review of Molecular Biology of the Cell).*TIBS* **18**, 147-148.

Gautier J.(1993). The cdc25 Protein Tyrosine Phosphatase Family. *Adv. Prot. Phos.* **7**, 151-179.

Solomon M., **Gautier J.**, Lee T. and Kirschner M. (1992). Control of p34^{cdc2} activation. Cold Spring Harbor Symposium on Quantitative Biology. Vol **56**, 427-436.

Gabrielli B., Roy L., **Gautier J.**, Philippe M. and Maller J. (1991) A cdc2.related kinase required for meiotic arrest in eggs oscillates in the cell cycle independently of cyclin and cdc2. *J. Biol. Chem.*, **267**, 1969-1975.

***Gautier J.**, Solomon M., Booher R., Bazan F. and Kirschner M. W. (1991). Cdc25 is a specific tyrosine phosphatase that activates p34^{cdc2}. *Cell.* **67**, 197-211.
* See comments. **Gautier J**. (1993) *The Scientist*, Hot Papers. May 17, 1993.

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Gautier J., Matsukawa T., Nurse P. and Maller J. (1989) Dephosphorylation and activation of *Xenopus* p34^{cdc2} protein kinase during the cell cycle.*Nature*, **339**,626-629.

***Gautier J.**, Norbury C., Lohka M., Nurse P. and Maller J. (1988) Purified maturation promoting factor contains the product of a *Xenopus* homolog of the fission yeast cell cycle control gene *cdc2*$^{+}$. *Cell* , **54**,433-439.
* See comments. **Gautier J**.(1990) *The Scientist*, Hot Papers. Feb 5, 1990.